RAG-1
13853

Efficacy of Stored TM BioControl-1®
and Research Related to its Use
in Suppression Programs

B. Kukan and I.S. Otvos

Natural Resources Canada
Canadian Forest Service

Ressources naturelles Canada
Service canadien des forêts

U.S. Department of Agriculture
Forest Service

Efficacy Of Stored TM BioControl-1® and Research Related to its Use in Suppression Programs

FINAL REPORT

Collaborative Research Agreement Between
the Canadian Forest Service
and
USDA Forest Service

Cooperative Agreement Number 00-11244225-026
(formerly 42-98-0014-A1)

B. Kukan and I.S. Otvos

Natural Resources Canada
Canadian Forest Service
Pacific Forestry Centre
506 West Burnside Road
Victoria, B.C. V8Z 1M5
CANADA

December 2001

Table of Contents

Executive Summary....ii

Introduction....v

Acknowledgements....ix

Section 1: Efficacy or shelf-life of stored TM BioControl-1®....1

Section 2: Testing potential anti-feedant properties of the carrier 038a® on the Oregon strain of Douglas-fir tussock moth....45

Section 3: Comparison of laboratory response of second-instar and third-instar larvae of Douglas-fir tussock moth to the Douglas-fir tussock moth using the diet surface contamination and diet plug methods....57

Section 4: Testing TM BioControl-1® infection of Douglas-fir tussock moth larvae reared on Douglas-fir foliage post-inoculation....79

Section 5: Effects of long-term storage on the stability of OpNPV DNA contained in TM BioControl-1®....91

Section 6: Acre-dose calculations....127

Section 7: Incidence of egg parasitism, level of naturally occurring virus, and egg viability in field collected Douglas-fir tussock moth egg masses from California, Oregon, Idaho, and Washington, 1998-2001....131

Section 8: Conclusions and recommendations....151

Executive Summary

This is the final report of the original Cooperative Agreement between the United States Department of Agriculture Forest Service and Natural Resources Canada, Canadian Forest Service.

The original aim of this cooperative project, running from July 1, 1998 to July 1, 2001, was to determine the efficacy of stored TM BioControl-1®. TM BioControl-1® is a biological insecticide that was registered, produced and stockpiled by the USDA Forest Service for the purpose of suppressing outbreaks of the Douglas-fir tussock moth (*Orgyia pseudotsugata* [McDunnough]).

The active ingredient in TM BioControl-1® is the naturally occurring nucleopolyhedrovirus of the Douglas-fir tussock moth (OpNPV) that is highly effective in controlling periodic outbreaks of this important defoliating pest of forests in western North America.

Additional studies evolved from this initial goal of determining the efficacy of stored TM BioControl-1® as work progressed over the 3 years of the project. These studies relate to the effective use of TM BioControl-1® to suppress Douglas-fir tussock moth populations. Results of these additional bioassays are all included in the report. The contents of the report are grouped into sections in an order that forest managers and administrators, faced with a control decision, might find useful.

Section 1. Bioassays conducted to determine the efficacy of stored TM BioControl-1® have shown that TM BioControl-1® stored at –10°C for 5-15 years is still effective, although it lost about 1/3 of its effectiveness during storage. This loss in potency does not appear to be directly related to length of time in storage. The size of the package, in which TM BioControl-1® was stored, does not appear to affect potency. There were no clear, significant differences in the efficacy of the various lots of the product processed by the four different companies. The bioassays showed that there are significant differences in the susceptibility of Douglas-fir tussock moth from the different geographic regions to TM BioControl-1® infection.

Section 2. The carrier (038a®) had no anti-feedant properties when it was used to deliver the virus contained in TM BioControl-1®, and in fact it may have some feeding stimulant properties. Carrier 038a® is suitable for use with TM BioControl-1® in Douglas-fir tussock moth suppression projects.

Section 3. The bioassay responses of second- and third-instar larvae to TM BioControl-1® in the laboratory were compared. Several conversion factors are given for forest managers to estimate lethal dose values for these two instars in control projects.

Section 4. The susceptibility of Douglas-fir tussock moth larvae to TM BioControl-1® infection is not affected in the laboratory when the larvae feed on newly flushed Douglas-fir foliage following diet-plug inoculation. Bioassays done in the laboratory using artificial diet give good indications of the efficacy of the virus against Douglas-fir tussock moth larvae in the field.

Section 5. DNA analysis of the virus contained in TM BioControl-1® showed that no detectable degradation, mutation or genetic change occurred in the Douglas-fir tussock moth viral DNA in any of the stored lots of TM BioControl-1®. Long term storage (5-15 years) at –10°C has not affected the structure of the viral genome and it is identical to the original standard (MEM-75 STANDARD) from which the various lots were produced. A contaminant (CPV) was detected in three of the sub-lots (4a, 5b and 6). The source of this contaminant is not known. The presence of OpCPV would be expected to enhance the pathogenicity of OpNPV in those three sub-lots.

Section 6. Based on bioassays conducted over a 3 year period (1998-2001), the acre-dose values (the amount of TM BioControl-1® to use per acre in a suppression project) for the 10 lots of TM BioControl-1® in storage were re-calculated and are presented along with a short-cut method for acre-dose calculation. This short-cut method should **not** replace bioassaying field populations of the target insect with the appropriate virus lot slated for use in the suppression project.

Section 7. This section provides data on egg parasitism, larval hatch, and level of naturally occurring virus from four western states (California, Oregon, Washington and Idaho). Although this was not part of the cooperative project, the results are included because they may help forest managers in their decision making when Douglas-fir tussock moth suppression projects are considered in the future.

Section 8. In this section the results of the 3-year Cooperative Agreement are briefly summarized. General recommendations are also made for future work on the Douglas-fir tussock moth and related areas.

Several sections of this report will be published in scientific journals.

Introduction

From the early 1980s to 1995 the United States Department of Agriculture, Forest Service (USDA Forest Service) undertook an ambitious project to produce and store Douglas-fir tussock moth nucleopolyhedrovirus (OpNPV) for use against the Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunnough) (Lepidoptera: Lymantriidae). The product was registered for use in the United States in 1976 as TM BioControl-1® (Martignoni 1978, 1999) and for use in Canada in 1983 (Otvos et al. 1998). Ten lots of OpNPV were produced between 1985 and 1995 and have been stored at the facilities of the USDA Forest Service in Corvallis, Oregon. Considerable time has elapsed since the original production and storage of the various TM BioControl-1® lots. In the summer of 1998, a Collaborative Project was initiated between the USDA Forest Service and Natural Resources Canada, Canadian Forest Service (NRCan-CFS), to test the efficacy or shelf life of the stored TM BioControl-1® product.

The original aim of this cooperative project was to test the efficacy or shelf-life of the stored TM BioControl-1®. Additional studies evolved from this main or initial goal as work progressed over the 3 years of the project as it relates to its effective use to manage Douglas-fir tussock moth populations in different regions in the western US, and these are all included in the report. This is the final report of the findings of the original Collaborative Agreement.

This report is divided into eight sections arranged in a way in which forest managers, and others, including those in administration, might find most useful and practical.

Section 1: Efficacy or Shelf-life of Stored TM BioControl-1®. The first section describes the bioassay work done to determine the current (1998-2001) efficacy of the stored TM BioControl-1®. This section also considers the effects of field strain of tussock moth (i.e. the susceptibility of Douglas-fir tussock moth populations to TM-BioControl-1® in different geographic areas), package size of stored product, length of time in storage, and the company that processed the virus killed larvae.

Section 2: Testing Potential Anti-feedant Properties of the Carrier 038a® on the Oregon Strain of Douglas-fir Tussock Moth. During the time of the Collaborative Agreement, a suppression project using the TM Biocontrol-1® was undertaken in Oregon in 2000. We were asked to test the potential anti-feedant properties of the carrier 038a® on the Oregon strain of Douglas-fir tussock moth. Our findings aided the suppression team to feel confident that the carrier 038a® was suitable for use with TM BioControl-1® against the Douglas-fir tussock moth.

Section 3: Comparison of Laboratory Response of Second-instar and Third-instar Larvae of Douglas-fir Tussock Moth to the Douglas-fir Tussock Moth Nucleopolyhedrovirus (OpNPV) using the diet surface contamination and diet plug methods. One question that arose during our studies involved the response of second and third instar Douglas-fir tussock moth larvae to the stored TM BioControl-1®. Often in the field there is a mix of instars and it is important to know the response differences in determining the dose or amount of product to be applied. We addressed this issue in this section by comparing the response of second and third instars using both a diet plug inoculation method and a diet surface contamination method. The comparison of these two inoculation methods and the use of the two larval instars also permitted a comparison between our bioassay method (diet plug with third instar) and that used earlier (diet surface contamination with second instar).

Section 4: Testing TM BioControl-1® Infection of Douglas-fir Tussock Moth Larvae Reared on Douglas-fir Foliage Post-Inoculation. It was felt that in addition to the response of larvae to TM BioControl-1® in the diet-reared insects, testing should include the response of larvae under more field-like conditions, i.e. the effect of feeding on foliage after inoculation. This testing was necessary to verify the effects of the stored product. In this section, we describe the experiment we conducted using Douglas-fir foliage as food after inoculation.

Section 5: Effects of Long-term Storage on the Stability of OpNPV DNA Contained in Stored TM BioControl-1®. This section deals with the question of whether or not storage for 5-15 years caused any genetic change in the viral DNA in the stored TM BioControl-1®, i.e. did storage cause any change in viral DNA in the product? DNA was purified and RFLP profiles were done on 17 samples from the 10 different lots of stored TM BioControl-1®. No degradation of viral DNA in TM BioControl-1®, stored for up to 15 years, occurred.

Section 6: Acre-Dose Calculations. In this section we discuss how to calculate acre-dose values of the stored TM BioControl-1® product. We also provide a short-cut method to do this and we have calculated the new acre-dose values for the stored product to assist forest managers in determining appropriate amounts of the stored product to use for Douglas-fir tussock moth suppression.

Section 7: Incidence of Egg Parasitism, Level of Naturally Occurring Virus, and Egg Viability in Field Collected Douglas-fir Tussock Moth Egg Masses from California, Oregon, Idaho, and Washington, 1998-2001. We have determined the incidence of egg parasitism and level of naturally occurring virus in field collected egg masses of Douglas-fir tussock moth from California when a suppression project was considered in the Sequoia and Kings Canyon National Parks in 1999. The same parameters were determined, along with egg viability, in the Oregon population when control measures were considered there against the Douglas-fir tussock moth in 2000. We have also determined egg viability, percent parasitism, and the level of naturally occurring virus incidence in Douglas-fir tussock moth populations from Oregon, Idaho and Washington when suppression projects were considered in these three states in 2001. Although this was not part of the cooperative project, we have included the results of these determinations from 1999 to 2001 to see if a pattern or change over time is revealed in the incidence of naturally occurring OpNPV, percent egg parasitism, and egg viability. These results may help forest managers in their decision-making when Douglas-fir tussock moth suppression projects are contemplated in the future.

There is some duplication in the Materials and Methods of some of these sections. We did this on purpose to save time either for the forest managers and/or for researchers should they wish to duplicate the experiments described in the sections. Each of the seven sections was written as a stand-alone unit so the readers do not have to search for essential or explanatory information in the other sections, something that would have been necessary if we cross-referenced parts of the various sections.

The plan is to publish as many of these sections in scientific journals as possible, because the peer review process will provide additional credence to the results and conclusions of the work that may be useful should future, planned suppression projects for Douglas-fir tussock moth populations be challenged in the courts.

Acknowledgements

There were many people involved in the various sections of this report and without their dedication the project could not have been completed. We thank Anne Van de Raadt, Meaghan Complin, Holly Douglas, Monika Fazekas, Andrea Fritz, Aster Mesfin, Andrea Quan, Karolina Monge-Monge, and Nicholas Conder for their technical assistance in the finicky, labor-intensive bioassaying. Holly Williams helped in completing Section 5. Joanna McGarvie helped with numerous revisions, with a cheerful smile, that made the report what it is. The illustration on the title page is the creation of Canadian Forest Service graphics artist Mark Lindal. Our sincere thanks go out to all of them for their help in making all this happen. We also thank Roy Magelssen for sending us the original bioassay data and Don Scott for reviewing earlier drafts of some parts of these sections. Funds were jointly provided under the cooperative research agreement between the USDA Forest Service, Forest Health Technology Enterprise Team, Morgantown, WV, USA, and Natural Resources Canada, Canadian Forest Service, Pacific Forestry Centre, Victoria, BC.

Section 1

Efficacy or Shelf-life of Stored TM BioControl-1®

B. Kukan[1] and I.S. Otvos[1]

[1] Natural Resources Canada, Canadian Forest Service, Pacific Forestry
Centre, Victoria, BC, V8Z 1M5, Canada.

Introduction

TM BioControl-1® is a viral insecticide that was produced and registered in 1976 by the United States Department of Agriculture (USDA) Forest Service for the control of Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunnough), outbreaks. Production of TM BioControl-1® occurred at the USDA Forest Service facility in Corvallis, Oregon (Martignoni 1978, 1999, Hadfield and Magelssen 1995). Fifth instar Douglas-fir tussock moth larvae of the Goose Lake laboratory strain (GL-1 strain), were infected with the Douglas-fir tussock moth virus, OpNPV, reared until death, and the cadavers were harvested and frozen. Over the course of 9 years, three private companies, Reuter, Espro and Crop Genetics, were contracted to process the virus-killed larvae and remove excess insect debris. The cadavers were lyophilized to remove moisture and milled to pass a 100-mesh screen resulting in a high potency, small particle size, virus-containing powder. The finished product was a wettable powder meeting the requirements of the registered product TM BioControl-1® and capable of being mixed in the field as an insecticide, and sprayed through ground and aerial spray equipment. The product had to be easy to mix and use under field conditions where control measures were required. The powder was vacuum-sealed in packages suitable for long-term storage at –10°C at the facilities of the USDA Forest Service Forestry Sciences Laboratory in Corvallis, OR. The Goose Lake colony, which originated from field collected Douglas-fir tussock moth egg masses from Northern California, has been maintained in the laboratory under controlled conditions since the mid-1960s at Corvallis up to 1995, and after that at the Pacific Forestry Centre (NRCan-CFS) in Victoria, BC.

The activity or infectivity titre of the processed product was determined by the USDA Forest Service on advanced samples provided by the processing companies. The bioassays were conducted at Corvallis using the diet surface contamination technique (Martignoni and Iwai 1977). Second instar larvae of the Goose Lake colony of the Douglas-fir tussock moth were exposed to several different concentrations of the virus sample being tested. The virus dilutions were applied to the surface of the artificial diet (Thompson and Peterson 1978) to determine how much of the preparation was required to cause 50% mortality in the test insects (median lethal concentration or LC_{50}) (Martignoni 1978, 1999; Martignoni and Iwai 1977, 1978).

The USDA Forest Service produced and stored 10 lots of TM BioControl-1® between 1985-1995 (Table 1). These lots were stored in 1,243 packages under vacuum and contained the equivalent of approximately 400,000 acre-doses (Hadfield and Magelssen 1995). From these packages, 47 samples, representing the 10 lots, were selected and sent to NRCan, Canadian Forest Service, Pacific Forestry Centre, Victoria, BC, for efficacy testing under a Cooperative Agreement. This Cooperative Agreement was between the USDA Forest Service and NRCan-CFS. The selection of samples was based on lot number, package size and time in storage (Table 1). The effects of the following variables were investigated in connection with the efficacy of the stored product:

A) Potency against some field strains of the Douglas-fir tussock moth from different geographical regions and the rest against the Goose Lake strain.

B) Package size.

C) Time in storage.

D) Company processing the virus killed insects.

The results and discussion for each of these topics will be presented under the above headings separately.

Table 1. List of the 20 g samples from the TM BioControl-1® packages in storage at the USDA Forest Service facility in Corvallis, OR, sent for efficacy determination to Natural Resources Canada, Canadian Forest Service, Pacific Forestry Centre in Victoria, BC

Lot #	Processed by	g/pkg	# pkgs	Total acre-dose determined at storage	Sample numbers	Date stored
1	USDA Agricultural Research Service	1,200	1	480	1	1985
2	Reuter	2,150	18	18,000	2, 3, 4	1986
3	Reuter	1,830	13	13,000	5, 6, 7	1986
4a[a]	Reuter	368	124	24,800	8, 10, 12	1986
4b[a]	Reuter	1,840	33	33,000	9, 11, 13	1986
5a[a]	Reuter	368	54	10,800	14, 16, 18	1986
5b[a]	Reuter	1,840	26	26,000	15, 17, 19	1986
6a	Espro	47	170	17,000	20, 21, 22	1989
6b[b]	Espro	180	1	383	None was sent	1989
7a	Espro	31.7	483	48,300	23, 27, 29	1990
7b	Espro	158.5	100	50,000	24, 26, 30	1990
7c	Espro	317	50	50,000	25, 28, 31	1990
8a	Crop Genetics	202	1	690	32	1991
8b	Crop Genetics	293	68	68,000	33, 34, 35	1991
9a[b]	Crop Genetics	49	1	84	None was sent	1993
9b	Crop Genetics	58	57	5,700	36, 38, 40	1993
9c	Crop Genetics	580	9	9,000	37, 39, 41	1993
10a	Crop Genetics	124.7	10	1,000	42, 44, 46	1995
10b	Crop Genetics	1,247	24	24,000	43, 45, 47	1995
Total			1,243	400,237[c]		

---

[a] Note that lots 4a and 5a were stored in identical sized packages (368 g/pkg), as were lots 4b and 5b (1,840 g/pkg).

[b] Lots 6b and 9a had only one package each (383 and 84 acre-doses, respectively); therefore, no sample was taken from these for efficacy evaluation after storage.

[c] The total acre-dose in storage was 400,237 prior to any suppression in 2000 and 2001.

Materials and Methods

Bioassay Protocol

The purpose of the bioassays was to determine the current activity titre (potency), or amount of activity per unit weight of preparation of the stored TM BioControl-1® samples against Douglas-fir tussock moth larvae. The potency was measured as the amount of preparation that causes the death of 50% of the test population of the larvae after 14 days. While we ran our bioassays for 21 days, because there were no significant differences in mortality at day 14 and day 21 we chose the shorter one for comparison. Activity standardization bioassays are the first step in the process needed for acre-dose determination (Martignoni 1978, Scott 2000). Each of the 10 production lots of TM BioControl-1® have a potency or activity titre that equates to an acre-dose treatment, based on bioassays with the Goose Lake strain, and is expressed as the activity units per gram (AU_{GL}/g) (Martignoni 1978, Scott 2000). After determination of the potency of the product in the laboratory with the Goose Lake strain or colony, the acre-dose (the amount of virus preparation to be used to treat 1 acre of Douglas-fir tussock moth infested stands) can be calculated from the second or acre-dose bioassay data for field application. Unlike the activity standardization studies, the acre-dose bioassays require Douglas-fir tussock moth larvae from the target field population (the population to be treated) and TM BioControl-1® from the lot(s) to be used in that particular suppression project. These two bioassays ideally should be done each time a new field population is scheduled for treatment in a new area and whenever a new lot of the product is to be used.

To obtain Douglas-fir tussock moth larvae for activity standardization bioassays, field-collected or Goose Lake colony Douglas-fir tussock moth egg masses (when no field-collected egg masses were available) were gently teased apart and the eggs were washed three times with 1% sodium hypochlorite (2% bleach) solution to reduce potential virus contamination, rinsed three times with distilled water, and then allowed to air dry. The decontaminated eggs were placed in sterile petri dishes and larvae reared at 25±1°C, 50-60% RH with a photoperiod of 16:8 (L:D). Larvae were reared in groups of 10 on artificial diet (Thompson and Peterson 1978). Newly molted third instar larvae (<24 h old) were selected from the petri dishes to standardize the age of the test larvae and were starved for 16-20 h before the bioassays.

Virus Preparation

Tap water usually contains chlorine, a chemical generally known to degrade viral particles. To avoid possible inactivation of the virus particles by tap water, 20 mg of the stored TM BioControl-1® sample to be tested was weighed out and prepared as a dilute slurry with 20 ml of distilled water. Samples were stirred on a magnetic stirrer for 2 h and viral samples prepared in triplicate. One week prior to inoculation, serial dilutions were made of the virus and stored at 4°C until they were used to infect the larvae in the bioassays. Concentrations were chosen to determine as accurately as possible the median lethal dose (LD_{50}) and that generally ranged from 16-200 polyhedral inclusion bodies (PIBs) per larva. Ideally, concentrations are selected to give approximately the LD_{30}, LD_{35}, LD_{45}, LD_{70} and LD_{75}, of the recommended lethal dose values to aim for in an accurate LD_{50} determination (Robertson and Preisler 1992).

The USDA Forest Service had quantified the polyhedral inclusion bodies (PIBs) concentrations of the TM BioControl-1® samples prior to storage using the haemocytometer method (Kalmakoff 1980). These counts were available in the original bioassay data provided to us by Mr. R. Magelssen, USDA Forest Service (Hadfield and Magelssen 1995). However, as a check, we have done haemocytometer counts of polyhedral inclusion bodies (PIB) on five randomly selected samples of the TM BioControl-1® samples to be tested: sample #2 from lot 2, #20 from lot 6, #36 and #37 from lot 9, and #43 from lot 10 (Table 2). The current PIBs/ml calculated at the start of this cooperative agreement were the same as the original PIB counts done at Corvallis, therefore, it was deemed unnecessary to recount the remaining 42 samples sent for bioassay. Serial dilutions were based on the original PIB counts.

A fresh OpNPV virus sample, to be used as a positive control, was prepared prior to the bioassay, by homogenizing in 5 ml of distilled water 100 Goose Lake strain Douglas-fir tussock moth larvae previously inoculated with OpNPV from lot 1 (sample #1) and reared until death. The fresh homogenate was filtered through cheesecloth, centrifuged once at 10 x g for 5 min and twice at 7100 x g for 20 min on an IEC centrifuge and resuspended in distilled water. PIB counts were done using a haemocytometer to quantify the PIB counts of the virus. The fresh OpNPV sample was produced 1-2 weeks prior to use, serial dilutions prepared and stored at 4°C until use in bioassays. None of the serial viral dilutions were kept longer than 3 months.

Table 2. Samples of stored TM BioControl-1® of the 10 lots bioassayed using third instar Douglas-fir tussock moth larvae (GL-1 strain) in diet plug experiments at Pacific Forestry Centre in Victoria, BC

Lot #	Package size[a]	g/pkg	TM BioControl-1® sample #	Total acre-dose (determined at storage)	Date stored
1	Large	1,200	1(2X)	480	1985
2	Large	2,150	2 3 4	18,000	1986
3	Large	1,830	5 6 7	13,000	1986
4a	Medium	368	8 10 12[b]	24,800	1986
4b	Large	1,840	9 11 13	33,000	1986
5a	Medium	368	14 16 18	10,800	1986
5b	Large	1,840	15 17 19	26,000	1986
6a	Small	47	20 21 22	17,000	1989
6b	Medium	180	None was sent (0)	383	1989
7a	Small	31.7	23 27 29	48,300	1990
7b	Medium	158.5	24 26 30	50,000	1990
7c	Medium	317	25 28 31	50,000	1990
8a[c]	Medium	202	32[c]	690	1991
8b	Medium	293	33 34 35	68,000	1991
9a	Small	49	None was sent (0)	84	1993
9b	Small	58	36 38 40	5,700	1993
9c	Medium	580	37 39 41	9,000	1993
10a	Medium	124.7	42 44 46	1,000	1995
10b	Large	1,247	43 45 47	24,000	1995
Total	17		39	400,237	

[a] Package sizes were defined as: small <100g/pkg (n=3, each of different sizes), medium 100-999g/pkg (8 of 9 are of different sizes), large >1000g/pkg (5 of 6 are of different sizes).

[b] Highlighted samples were not bioassayed because the LD_{50} of the other two samples from the same lot were not significantly different. Therefore, bioassaying the third sample was deemed unnecessary and would have been a “waste of money, time and effort”.

[c] Sample #32 came from lot 8a and represented only 690 acre-doses; therefore, it was not included in the analysis even though it was bioassayed.

Inoculation

The diet plug inoculation bioassay technique we used was similar to that of Kaupp and Ebling (1990). Only those larvae that eat the whole diet plug were used in the experiment. The use of diet plug bioassay is superior to diet surface contamination because it eliminates any variation in the distribution of PIBs on the diet surface and eliminates any differential feeding rates and its consequences among the larvae. When the diet surface contamination technique is used there can be differences in larval feeding rate, number of PIBs consumed and interference among larvae reared in the same container due to crowding. In our tests, either 1 μl of each viral dilution, or distilled water in the control, was added to a small diet plug (3-4 mg) inside each well of a 24-well tissue culture plate. Previous experiments have shown that the diet plugs were large enough to fully absorb the 1μl of liquid and small enough that one larva could generally consume it in 24 hours.

For each of the TM BioControl-1® samples, as well as the fresh OpNPV sample (positive control), five virus concentrations were tested. Bioassay of the five dilutions of each TM BioControl-1® sample, plus the fresh preparation of OpNPV, and an untreated or negative control (distilled water) were replicated three times.

The replicates were done on three successive days. Freshly molted third instar Douglas-fir tussock moth larvae, starved for 16-20 h before the bioassay, were used in our tests. Immediately after inoculation of the diet plugs, one third instar newly molted (<24 h old) larva was placed into each well to feed on the treated diet plug.

Larvae were held in darkness for 24 h with the inoculated diet plug at 25±1°C, 50-60% RH. Only larvae that consumed the entire diet plug were used in the bioassays and were placed in individual cups (Solo P100, Solo Cup Co. Urbana, Illinois 61801-2895) with fresh, untreated diet and returned to the same growth chamber. Larvae, which did not consume the entire diet plug, were discarded. Because of the high virulence of OpNPV, it was necessary to rear the larvae individually after inoculation to avoid cross-infection. In addition, control and viral infected insects were reared in separate growth chambers, set at the same rearing conditions, to guard against viral transmission to the control insects. Diet was changed at least weekly or more often, if the diet dried out. Larvae were reared for 21 days post inoculation and were monitored and the mortality recorded daily. Only larvae that died from NPV infection, as determined by gross pathology and verified by microscopic examination, were included in the analysis.

For each viral dilution or control 48 larvae were tested (Table 3). Each experimental run included five TM BioControl-1® samples, as well as the positive and negative controls and took 3-4 months to complete. Each experimental run required approximately 5,000 larvae. To insure that this number of larvae were available at the appropriate stage of development (i.e., 5,000 newly molted third instars over a 3-day period), 10,000 larvae had to be reared to select the 5,000 freshly molted third instar, which in turn would have to be reared from about 30,000 eggs to insure sufficient numbers of insects in each replicate.

Table 3. Number of third instar Douglas-fir tussock moth larvae used per replicate in each of the large bioassays testing the efficacy of stored TM BioControl-1® samples

Treatment Concentration of each sample tested[a]	# of third instar Douglas-fir tussock moth larvae used Replicate A	Replicate B	Replicate C
1	240	240	240
2	240	240	240
3	240	240	240
4	240	240	240
5	240	240	240
Fresh (positive control)	240	240	240
Control (distilled H_2O) (negative control)	48	48	48
TOTAL	1,488	1,488	1,488

The total number of Douglas-fir tussock moth larvae used in one large experimental run of bioassays were 1,488 x 3 = 4,464 larvae. There were 3 replicates.

[a] For each sample of stored TM BioControl-1®, one fresh virus (positive control), and one negative control (distilled water) were tested.

5 concentrations x 48 larvae / concentration = 240 larvae / sample

6 virus samples (five stored and one fresh) were tested in a large bioassay = 6 x 240 = 1,440 per replicate

1 control for each replicate with 48 larvae 1,440 + 48 = 1,488

The five concentrations were selected, ideally, to give approximately LD_{30}, LD_{35}, LD_{45}, LD_{70} and LD_{75} of the recommended lethal dose values (Robertson and Preisler 1992). 1= LD_{30}, 2=LD_{35}, 3=LD_{45}, 4=LD_{70} and 5=LD_{75}.

TM BioControl-1® Samples Selected for Testing

Of the 1,243 packages of TM BioControl-1® product stored at the USDA Forest Service facility in Corvallis, OR, 47 samples, each weighing 20g, were sent to the Pacific Forestry Centre in Victoria, BC for testing. The 47 samples sent to Victoria represented all 10 lots. The 47 samples were ranked in terms of time in storage, package size, and acre-dose (the amount of TM BioControl-1® in storage) available. It would have been too time-consuming, costly, and unnecessary to bioassay all 47 samples. Therefore, the 47 samples representing 17 different package types were grouped into three weight classes: small, medium and large. These were defined as: small <100 g/pkg (n=3), medium 100-999 g/pkg (n=8) and large >1000 g/pkg (n=6).

The bioassays were performed, initially, using larvae reared from field collected Douglas-fir tussock moth egg masses from California (CA), Oregon (OR), Idaho (ID), and British Columbia

(BC), and then exclusively with the Goose Lake laboratory colony (GL-1 strain) which has been maintained at the Pacific Forestry Centre since March 1996.

At the Year 1 evaluation meeting (August 15, 1999) attended by Drs. R. Reardon, I. Ragenovich. I.S. Otvos and B. Kukan, it was decided to complete the remaining bioassays using the Goose Lake colony. Goose Lake larvae were chosen for the comparisons of package size, time in storage, and possible differences caused by processing by the different companies because the first few bioassays, using field collected Douglas-fir tussock moth from CA, OR, ID and BC, indicated that geographic origin of the field strain of the Douglas-fir tussock moth affected the LD_{50} values (see Fig. 1 and 2 in the “Potency of Stored TM BioControl-1® Tested Against Different Strains of Douglas-fir Tussock Moth from Different Geographical Regions: Results” Section [pp. 13-16]). In addition, egg masses could be obtained more readily and reliably from laboratory rearing than from field collections, and the laboratory strain is also free of egg parasitism and the naturally occurring virus. The Goose Lake strain can also be “produced year round” in the laboratory with the appropriate cold storage to break diapause, thus providing a more “flexible” larval hatching schedule so more bioassays can be done in a shorter time. Furthermore, the original efficacy data used for registration was also obtained from tests conducted with the Goose Lake strain. Another benefit of using the Goose Lake strain in the remaining bioassays is that using this strain will make the re-registration of TM BioControl-1®, at a later date, easier.

The TM BioControl-1® samples, used in bioassays with the Goose Lake strain in seven large bioassay runs, are listed in Table 2 and Table 4. At least two of each package size were analyzed and more samples were done in lots that have large acre-doses of the product (Table 2).

Table 4. Total number of samples of the stored TM BioControl-1® tested from each lot in the bioassays with third instar Douglas-fir tussock moth larvae (GL-1 strain) using the diet plug inoculation method

Lot tested	Total number of samples tested
1	1 (#1 2x)
2	3 (#2 2x)
3	3
4	4
5	4
6	3
7	6
8	4
9	6
10	5
Total	39 + 2 repeats

Data Analysis

Using the larval mortality data, dosage mortality curves and LD_{50} values in PIBs with associated 95% fiducial limits were calculated using PROC PROBIT analysis (SAS Institute 1989-1996). Each replicate at day 14 post-inoculation (and day 21) was tested as a separate preparation to verify that there was no difference in the replicates. Because no differences were found, the data for the three replicates were combined. Estimates of LD_{50} for each TM BioControl-1® sample and the fresh OpNPV sample were calculated. LD_{50} values at day 14 and day 21 were compared to see if lethal doses changed over time. LD_{50} values were examined for significant differences (no overlap of the 95% fiducial limits).

Variation of LD_{50} among TM BioControl-1® samples were also examined. There were no significant differences ($p<0.05$) between LD_{50} values at day 14 compared to day 21 for 39 of the viral samples tested. Therefore, we used the day 14 data in the analysis.

Experiments were designed to calculate the median lethal dose (LD_{50}). Concentrations were selected to give approximately 30%, 35%, 45%, 70% and 75% mortality, as recommended by Robertson and Preisler (1992). For each bioassay, the concentrations chosen were based on previous experiments as a best guess of what was appropriate. However, the samples were not all the same and in rare cases the dilutions chosen did not give the desired mortality levels for the sample tested resulting in large error bars (at the $p<0.05$ level) as in sample #15 (Fig. 2) where the upper value of the fiducial limit is 269.8. The bioassays are costly and time consuming, and since two or more samples per package size per lot were tested, not all TM BioControl-1® samples were bioassayed or certain bioassays were not always repeated when the results of the two replicates agreed or were not significantly different. The TM BioControl-1® samples bioassayed are given in Table 2.

The efficacy of a fresh sample of OpNPV was compared among four different field strains of Douglas-fir tussock moth (California, Oregon, Idaho, B.C.) and the GL-1 (Goose Lake) laboratory strain. In addition, when sufficient numbers of larvae of these field strains were available, TM BioControl-1® samples were also tested with the fresh OpNPV sample. TM BioControl-1® samples tested with the CA strain were lot 4 (samples #8 and #9), lot 7 (samples #23 and #24), and lot 8 (sample #33). Idaho strain was tested with lot 2 (sample #3), lot 6 (sample #21), and lot 10 (sample #44). The BC strain was tested with lot 3 (sample #5) and lot 5 (samples #14 and #15) (Fig. 2). The low number of larvae that emerged from the egg masses received from Oregon in the first year (1999) only permitted testing fresh OpNPV.

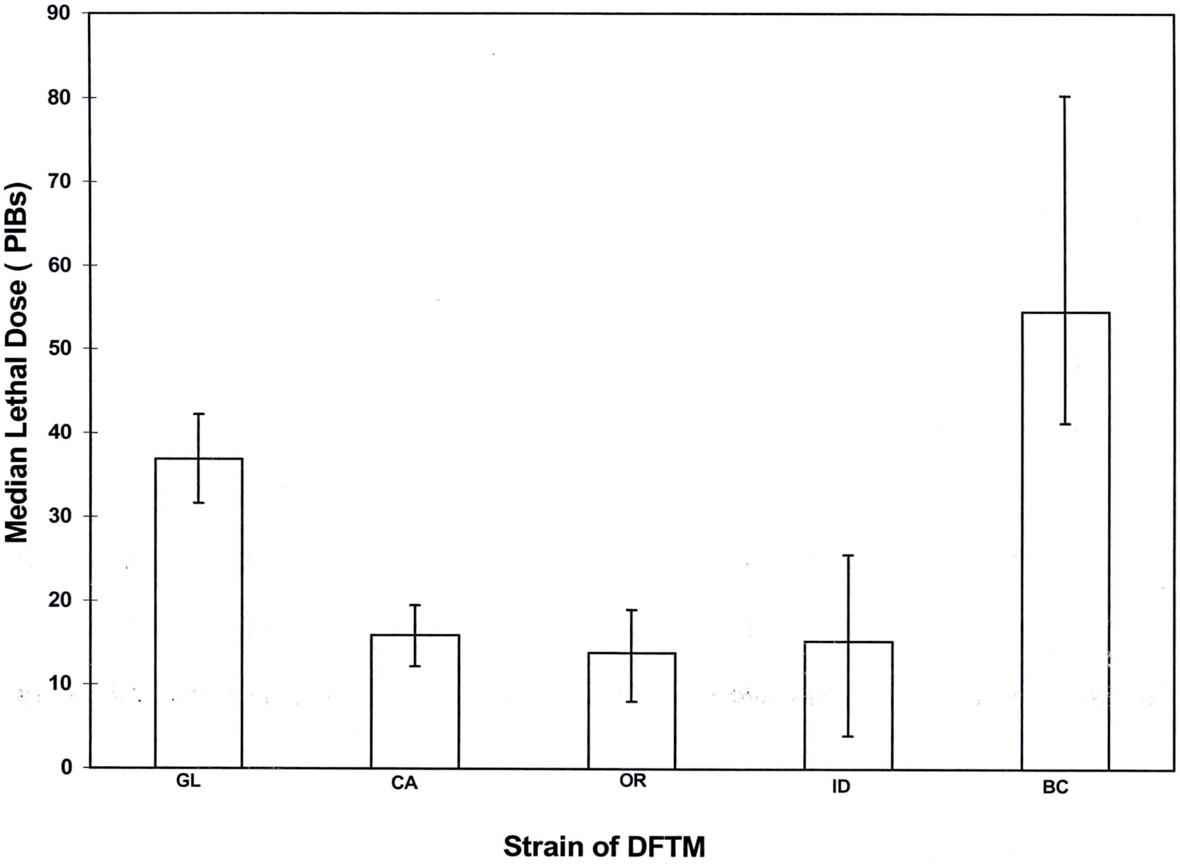
1) Potency of Stored TM BioControl-1® Tested Against Different Strains of Douglas-fir Tussock Moth from Different Geographical Regions.

Results

Results from challenges of different strains of Douglas-fir tussock moth with a fresh OpNPV sample indicate that there is some variation in LD_{50} values among the different strains of Douglas-fir tussock moth (Fig. 1). The three field strains from the US (CA, OR, ID) have similar, relatively low LD_{50} values when compared to the BC strain. The LD_{50} value of the BC strain is significantly higher, about three-fold higher with fresh OpNPV, even when overlap of the fiducial limits are compared ($p<0.05$). The LD_{50} values for the Goose Lake strain challenged with fresh OpNPV gave intermediate values, i.e. it was higher than for the three field strains from the US but lower than the LD_{50} value for the BC strain.

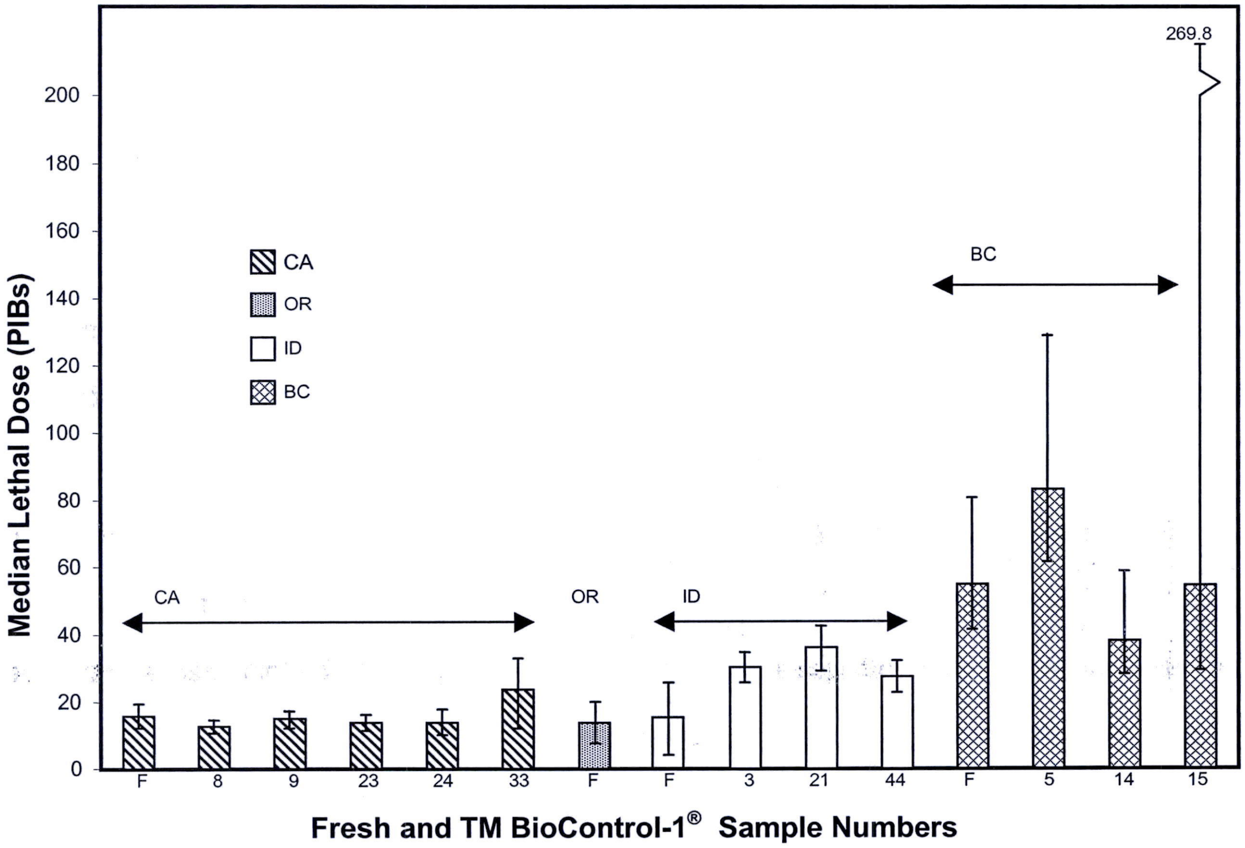
The mortality response of different strains of Douglas-fir tussock moth to TM BioControl-1® is shown in Figure 2. LD_{50} values among TM BioControl-1® samples tested are similar to that of the fresh OpNPV sample for the CA and BC strains (fiducial limits overlap). For the ID strain, the LD_{50} value for one TM BioControl-1® sample #21 of lot 6, was significantly higher ($p<0.05$) than for fresh OpNPV. Because different lots of TM BioControl-1® were used it is difficult to make comparisons and generalizations. Nevertheless, it is apparent that the three US strains (CA, OR, and ID) of Douglas-fir tussock moth appear to be more susceptible to TM BioControl-1® than the BC strain. Because the strain of Douglas-fir tussock moth used in the bioassays affects LD_{50} values, it was decided, in consultation with Drs. R. Reardon and I. Ragenovich, to use the Goose Lake strain from this point on to compare all TM BioControl-1® samples to assess the effects of time in storage, effects of package size and possible differences caused by processing by different companies. The original Goose Lake strain (GL-1 strain) was chosen for this, and reasons for using it in the bioassays have already been given in the section on "TM BioControl-1® Samples Selected for Testing" (pp. 10-12).

Fig. 1. Comparison of median lethal dose (LD_{50}) in PIBs of fresh OpNPV per third instar of different field strains and the Goose Lake (GL) strain of Douglas-fir tussock moth larvae.



[a] GL = Goose Lake laboratory colony.
Field strains: CA = California, BC = British Columbia, OR = Oregon, ID = Idaho.
Bars indicate 95% Fiducial Limits

Fig. 2. Comparison of the median lethal dose (LD_{50}) values in PIBs per third instar larvae of the different strains of Douglas-fir tussock moth tested with a fresh sample of OpNPV (F) and with stored TM BioControl-1®.[a]



[a] Douglas-fir tussock moth strains tested:

CA with stored TM BioControl-1® samples 8 and 9 (lot 4), 23 and 24 (lot 7), and 33 (lot 8).
ID with TM BioControl-1® sample 3 (lot 2), 21 (lot 6), and 44 (lot 10).
BC with TM BioControl-1® samples 5 (lot 3), 14, and 15 (lot 5).
All three of these strains were also challenged with freshly produced OpNPV.
OR was tested only with fresh OpNPV (F).
(Field strains: CA = California, BC = British Columbia, OR = Oregon, ID = Idaho)

Discussion

Our results indicate that the three US field strains of Douglas-fir tussock moth seem to be more susceptible to OpNPV than the BC strain of Douglas-fir tussock moth. Other researchers have also reported that the strain of insects can affect the susceptibility to nucleopolyhedrovirus (NPV). Milks (1997) reported a 3.5-fold difference in susceptibility of 12 lines of cabbage looper, *Trichoplusia ni* (Hubner) (*T. ni*), to the singly embedded nucleopolyhedrovirus of *T. ni* (TnSNPV). Similarly, Skatulla (1987) observed variations of mortalities ranging from 26.1% to 90.0% in gypsy moth, *Lymantria dispar* (L.), larvae from different geographical regions of Europe when the larvae were given the same concentration of NPV in the bioassays. Reichelderfer and Benton (1974) have found a 5-fold difference in the LD_{50} values of two strains of *Spodoptera frugiperda* (J.E. Smith), given the same doses of a nucleopolyhedrovirus. Similarily, Aratake(1973) found large variation in LD_{50} values among various strains of *Bombyx mori* (L.) challenged with NPV. Most authors suggest that a genetic difference in the insect strains could account for the differences in susceptibility to viruses. However, none of the studies provided proof that this was the case. In our study, the BC strain was less susceptible to OpNPV than the strains from the US. It is possible that the US strains are more genetically alike than the BC strain (where Douglas-fir tussock moth is at the northern limit of its distribution), which could account for differences in susceptibility, but we did not test for this, so we can only speculate.

Recommendation

Since there is variation in the median lethal dose values of different strains it is important and **necessary** to consider the target population in a suppression or control project. The amount of TM BioControl-1® available is limited. If a target field strain of Douglas-fir tussock moth is tested to be more susceptible then it may be possible to use less TM BioControl-1® per acre to achieve the desired levels of larval mortality and foliage protection. In anticipation of this, perhaps **reduced amounts of the approved virus dosage could and should be tested for efficacy under field**

conditions in the US. Reduced dosages of TM BioControl-1® were tested successfully in BC in 1982. The results in BC showed that the registered dose (2.5 x 10^{11} PIBs/ha) can be reduced to about 1/3 of this and still get high larval mortality (95% at full dose, 91% at 1/3 dose). For details of this reduced dose experiment in Canada see Otvos et al. (1987).

2) Package Size

Results

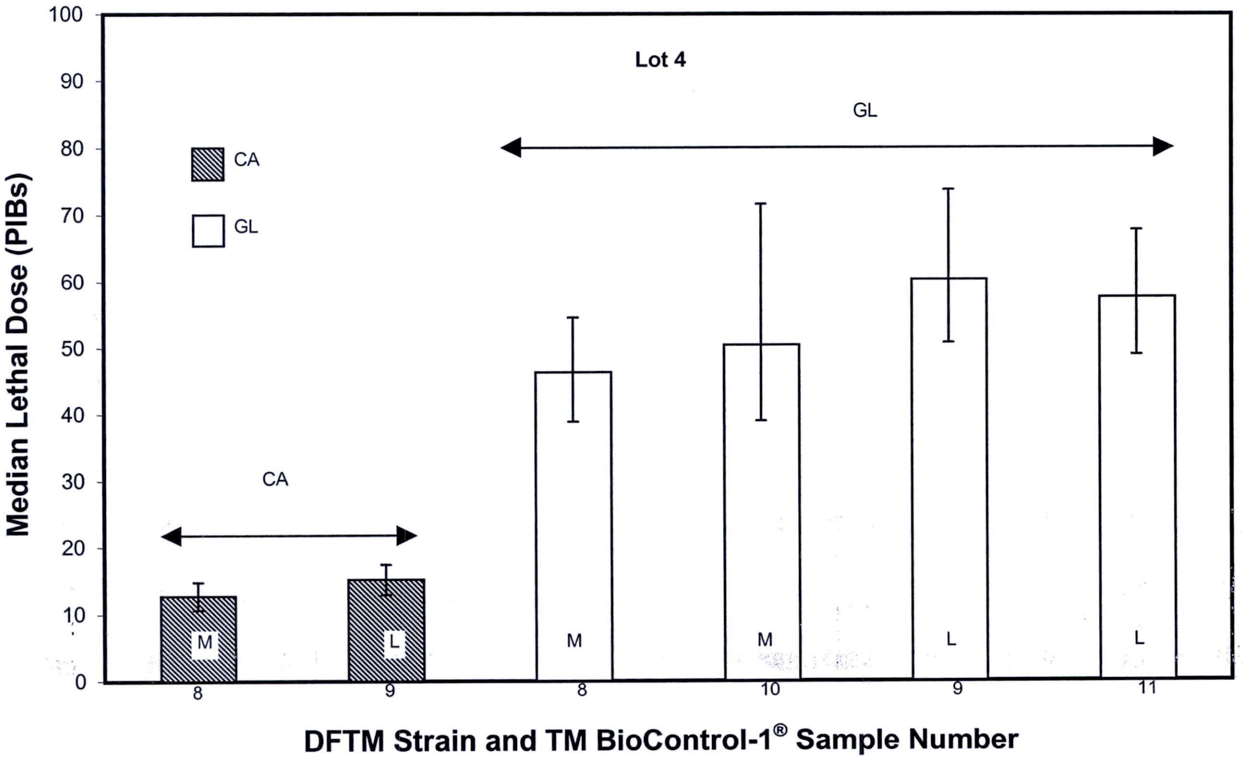
Ten lots of TM BioControl-1® were produced between 1985 and 1995 and were stored in a total of 1,243 packages (Table 1). These 10 lots were packaged in different package sizes ranging from 31.7 g/package to 2,150 g/package. Within lots, one, two or three different package sizes were produced, resulting in a total of 19 different package "types" (Table 1). The number of packages in each of these 19 different package types varied from 1 to 483. Within the 19 different package "types", two pairs of "types" were identical in weight (lots 4a and 5a [both 368 g/pkg], and lots 4b and 5b [both 1,840 g/pkg]); thus there were 17 different package sizes within the lots. These 17 package sizes were divided based on size into three groups for analysis. Packages less than 100 g/pkg were considered small, between 100-999 g/pkg were classed as medium, and >1,000 g/pkg were considered large packages. Among the packages included in the analysis, the small packages (n=3) ranged in weight from 31.7 - 58 g/pkg, the medium packages (n=7) from 124.7 - 580 g/pkg, and the large packages (n=6) 1,200 - 2,150 g/pkg (Table 1). Three additional package types, representing lot 6b, lot 8a, and lot 9a, had only one package each (383, 690, and 84 acre-doses, respectively), and were excluded from the analysis and thus the package size categories. No samples were taken from lot 6b and lot 9a, because they represented only 383 and 84 acre-doses, respectively, for efficacy evaluation. Although one sample was received from lot 8a, it was not included in the efficacy evaluation (although it was bioassayed) because of its relatively small, 690 total acre-dose size. It was considered to be more important to test samples from lots with higher total acre-doses.

Three of the five lots (4, 5, 10) had one medium and one large package size each, two lots (7 and 9) had small and medium packages, and the remaining three lots (1,2 and 3) were stored in large packages only (Table 1).

The comparisons of LD_{50} values from the different package sizes were done with three strains of the Douglas-fir tussock moth larvae. One comparison was made with the BC strain (sample 14 and 15 from lot 5, Fig. 4) and two with the California strain (samples 8 and 9 from lot 4, Fig. 3, and samples 23 and 24 from lot 7, Fig. 5). All other comparisons were done with the Goose Lake strain.

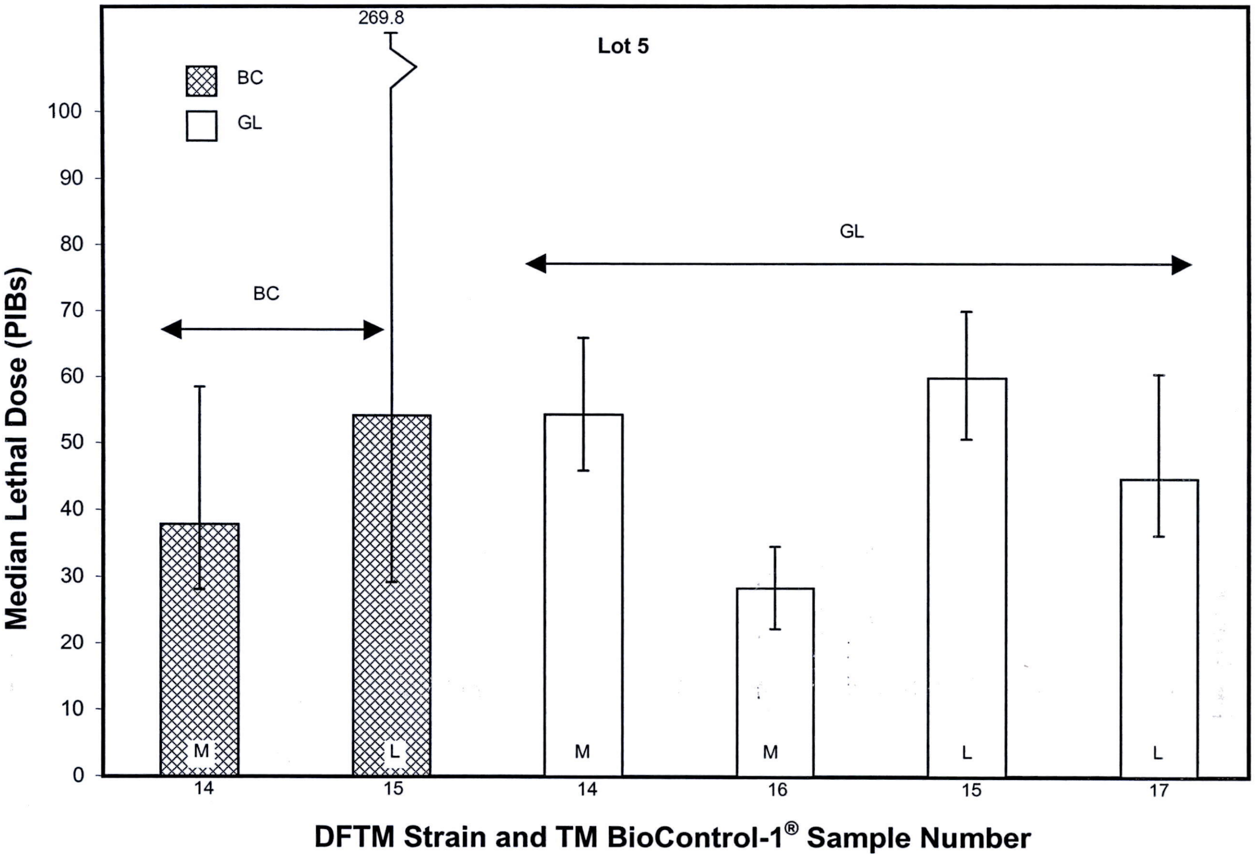
LD_{50} values of the samples bioassayed from the different package sizes but from the same lot are compared in Figures 3-7. Although two different (field and laboratory) strains of Douglas-fir tussock moth were used in some of the comparisons (see Fig. 3, 4 and 5), the effects of package size were **always** compared between and among the bioassays using the **same strain** of Douglas-fir tussock moth.

Fig. 3. Comparison of median lethal dose (LD_{50}) values, in PIBs per third instar Douglas-fir tussock moth larvae for the different package sizes in lot 4 of the stored TM BioControl-1®.[a]



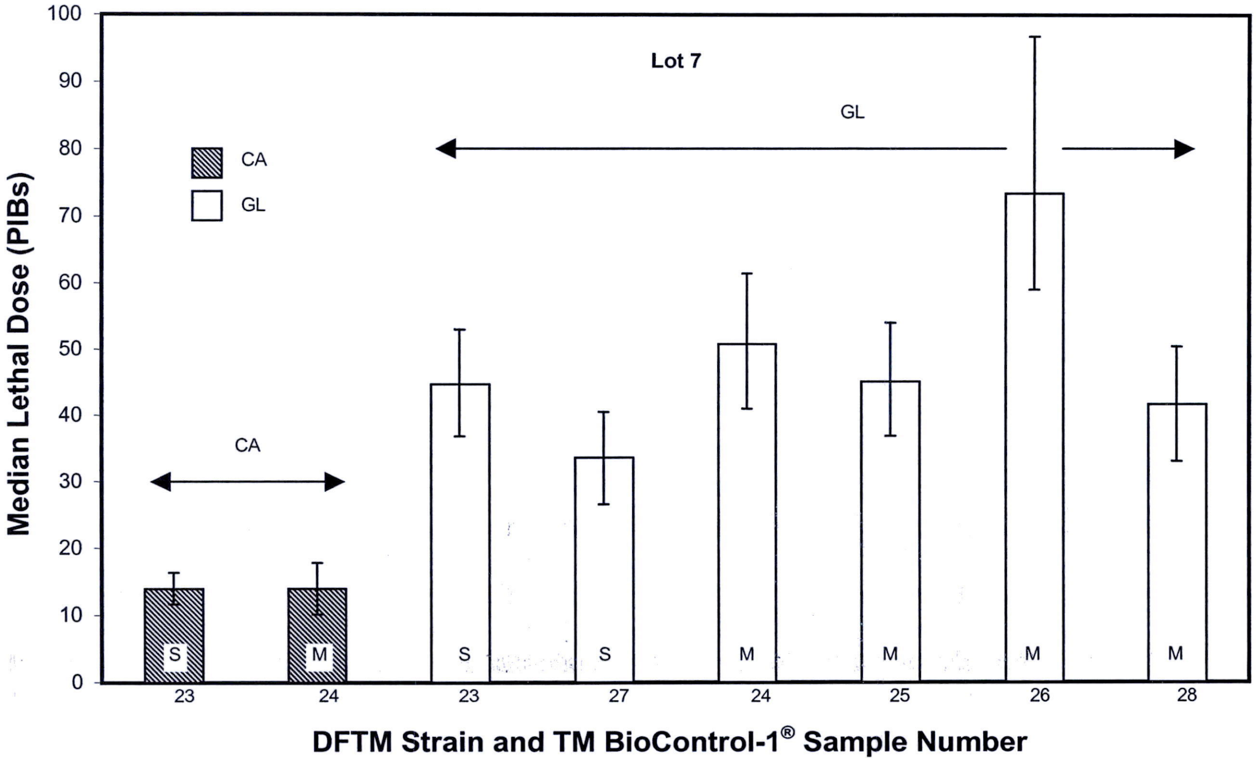
[a] Strains used: CA = California field strain, and GL = Goose Lake laboratory strain. Package sizes were M = medium 100-999 g/pkg., and L = large > 1000 g/pkg. Numbers under the bar graphs denote sample numbers of the stored virus product.

Fig. 4. Comparison of median lethal dose (LD_{50}) values, in PIBs per third instar Douglas-fir tussock moth larvae, for the different package sizes in lot 5 of the stored TM BioControl-1®.[a]



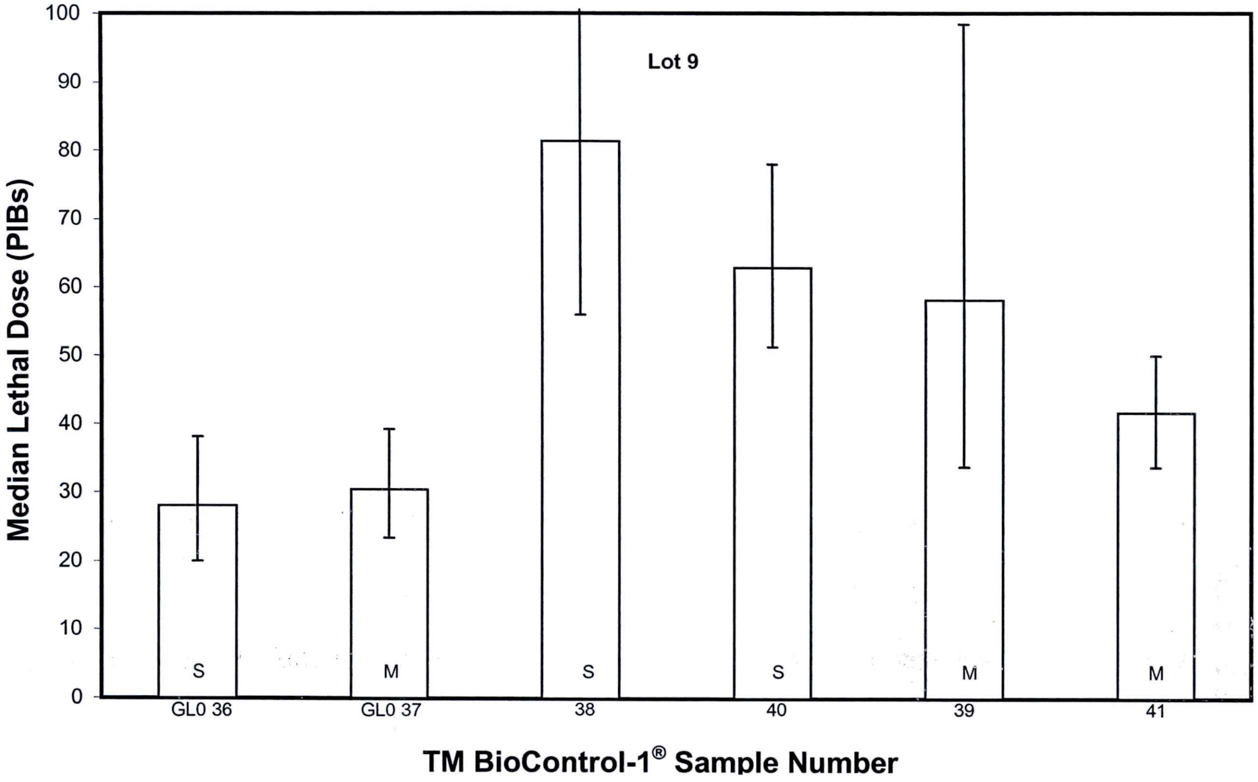
[a] Strains used: BC = British Columbia field strain, and GL = Goose Lake laboratory strain. Package sizes were M = medium 100-999 g/pkg., and L = large > 1000 g/pkg. Numbers under the bar graphs denote sample numbers.

Fig. 5. Comparison of LD_{50} values, in PIBs per third instar Douglas-fir tussock moth larvae, for the different package sizes in lot 7 of the stored TM BioControl-1®.[a]



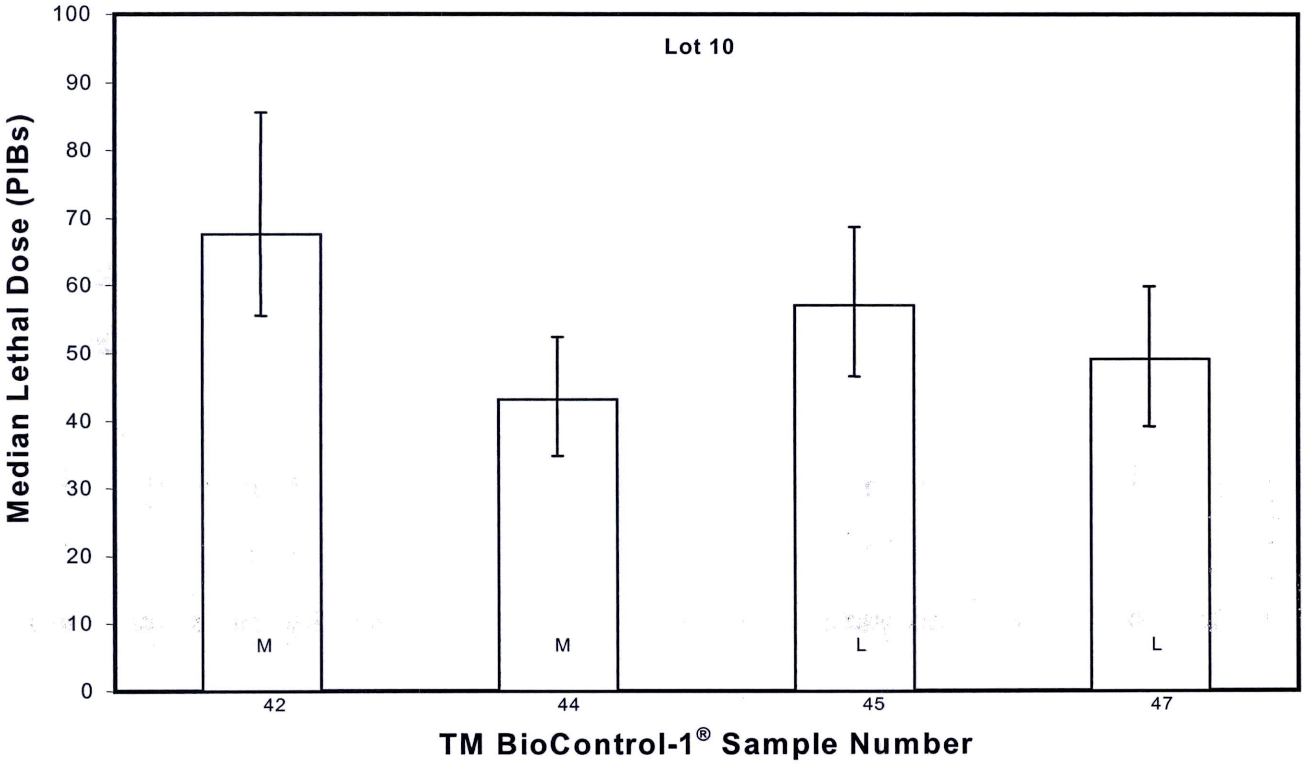
[a] Strains used: CA = California field strain, and GL = Goose Lake Laboratory strain. Package sizes were S= small < 100 g/pkg., and M = medium 100-999 g/pkg. Numbers under the bar graphs denote sample numbers.

Fig. 6. Comparison of LD_{50} values, in PIBs per third instar Douglas-fir tussock moth larvae, for the different package sizes in lot 9 of the stored TM BioControl-1®.[a]



[a] Strain used: GL = Goose Lake laboratory strain. GL0 was the first bioassay using the Goose Lake strain. Package sizes were S = small <100g/pkg., and M = medium 100-999 g/pkg. Numbers under the bar graphs denote sample numbers.

Fig. 7. Comparison of LD_{50} values, in PIBs per third instar Douglas-fir tussock moth larvae, for the different package sizes in lot 10 of the stored TM BioControl-1®.[a]



[a] Strain used: GL = Goose Lake laboratory strain. Package sizes were M = medium 100-999 g/pkg., and L = large > 1000 g/pkg. Numbers under the bar graphs denote sample numbers.

Medium and Large Packages. All but three of the comparisons were made with larvae of the Goose Lake strain of Douglas-fir tussock moth. A comparison of LD_{50} values between the medium and large packages in lot 4 (samples #8 and #9, respectively) showed no significant differences when the CA field strain of Douglas-fir tussock moth larvae were used for the comparison (Fig. 3). Similarly, there was no significant difference in LD_{50} for medium (#8, #10) and large packages (#9, #11) when the comparisons were made with the Goose Lake strain of Douglas-fir tussock moth. The LD_{50} values for both samples 8 and 9 tested with the field strain from California were significantly lower (i.e. this strain was more susceptible) than with the Goose Lake strain. For lot 5

(Fig. 4), there was also no significant difference when a medium package (sample #14) was compared to a large (#15) using the BC strain. Similarly, there was no significant difference for a medium package (#14) compared to large packages (#15 and #17) tested with the Goose Lake strain. However, the LD_{50} for the medium package (represented by #16) was significantly lower ($p<0.05$) than the LD_{50} value for the other medium package (#14) and both large packages (#15 and #17) using the Goose Lake strain (Fig. 4). We have no explanation for this difference, except that TM BioControl-1® in this package (sample #16) may have degraded faster or was already less effective at the time of packaging than in the other packages. However, there could be other explanations for this. It may be interesting to note that there was no significant difference between the medium size packages when sample #14 (bioassayed with the BC strain Douglas-fir tussock moth) is compared with sample #14 (bioassayed with GL) or sample #16 also bioassayed with GL. However, there is significant difference between samples #14 and #16 even though both were tested against GL strain.

In lot 10 (Fig. 7), the LD_{50} values obtained with the Goose Lake strain for a medium package (sample #44) are not significantly different from large packages (#45 and #47). Similarly, the LD_{50} value for a medium package (#42) was not significantly different from the LD_{50} values obtained from large packages (#45 and #47). However, the LD_{50} value of medium package (#42) is significantly higher ($p<0.05$) than medium package (#44). Again, we have no explanation for this difference.

Small and Medium Packages. In lot 7 (Fig. 5), there was no significant difference in LD_{50} values in the small package (sample #23) compared to the medium package (#24) when bioassays were done with CA strain. Similarly, in bioassays done with the Goose Lake strain, there was no significant difference in LD_{50} between small packages (#23 and #27) compared to medium packages (#24, #25 and #28). However, medium package (sample #26) had a significantly higher LD_{50} value ($p<0.05$) than small packages (#23 and #27) and was also higher than LD_{50} values obtained for medium packages (#25 and #28) but not for medium package (#24) with the Goose Lake strain of Douglas-fir tussock moth.

An interesting pattern can be noted here. There were no significant differences between the LD_{50} values of the small package (sample # 23) and the medium package (sample #24) compared with either the CA or Goose Lake strain, but the LD_{50} was significantly lower ($p<0.05$) in the bioassay done with the CA strain. This is expected since the CA strain appeared to be the most susceptible to TM BioControl-1® among the Douglas-fir tussock moth tested. This high susceptibility of the field strain of the Douglas-fir tussock moth from California could be due in part to the presence of another virus, indicated by preliminary analysis.

For lot 9 (Fig. 6), the LD_{50} of the small package (sample #36) was not different from the medium package (#37) in GL0 bioassay, which was the first bioassay conducted using the Goose Lake strain. The LD_{50} values for Goose Lake in the first experiment (GL0), done in the first year of the cooperative project, were generally lower than in the following seven large experiments (GL1-GL7). This may be due to a healthier colony and to some minor adjustment to the Douglas-fir tussock moth diet after the first bioassay. There was also no significant difference between small packages (samples # 38 and #40) and medium package (sample #39). Similarly, there was no significant difference in the LD_{50} values between the medium packages (samples #39 and #41). However, the LD_{50} value of medium package (sample # 41) was significantly lower ($p<0.05$) than the LD_{50} value for the small packages (sample #38 and #40) but not different from the LD_{50} value of medium package (sample #39). No comparison was made between the package sizes in lot 6 and 8 because each had only one type of package size, the former had only one package size (all small) while lot 8 also had only one package size (medium).

Discussion

All but three of the comparisons were made in bioassays using the Goose Lake strain of the Douglas-fir tussock moth. In two of these three cases the comparison was done using larvae of the California strain (Fig. 3 and Fig. 5), and in the third case larvae from British Columbia were used (Fig. 4). Because the comparisons of the different package sizes were done using the same strain even with the California and BC strain all of these comparisons are valid.

The results from the comparisons of LD_{50} values of different package sizes are summarized in Table 5. Fourteen comparisons were made between LD_{50} values obtained from testing samples from small and medium packages. Of these 14, 10 (or 71%) showed no significant difference in the LD_{50} values while the remaining four did. Fourteen comparisons were also made between LD_{50} values of medium and large packages. Of these 14, 12 (or 85.7%) showed no difference in the LD_{50} values. Overall, of the 28 different comparisons of LD_{50} values obtained for samples from small to medium and medium to large, 22 (or 78.6%) showed no difference in the LD_{50} values.

Three samples (#16, #26 and #41, all from medium package sizes) were involved in comparisons of package sizes where significant differences were observed. The LD_{50} in samples #16 and #41 was lower while in sample #26 it was higher than the LD_{50} values of their comparison samples. Because each of these samples was involved in two comparisons where there were significant differences, these three samples are suspect, i.e. probably do not represent the whole lot, but rather only the sub-lot from which they came. Because sample #16 (sub-lot 5a) was lower, as was sample #41 (sub-lot 9c), they should be used later from among the medium packages. Conversely, because sample #26 (sub-lot 7b) had a higher LD_{50} value this sub-lot should be used first, i.e. during the next suppression project.

There were no significant differences among the LD_{50} values of the samples from the large package sizes.

Table 5. Summary of the bioassay results comparing LD_{50} values obtained by testing samples from the various package sizes in the same lot of stored TM BioControl-1®

Lot #	Douglas-fir tussock moth strain	Samples compared	Significant difference	Comments
4	**CA**	8 M and 9 L	No	See Fig. 3
	GL	8 M and 9 L	No	See Fig. 3
	GL	8 M and 11 L	No	See Fig. 3
	GL	10 M and 9 L	No	See Fig. 3
	GL	10 M and 11 L	No	See Fig. 3
5	**BC**	14 M and 15 L	No	But high 95% F.I. for BC 15 (Fig. 4)
	GL	14 M and 15 L	No	See Fig. 4
	GL	14 M and 17 L	No	See Fig. 4
	GL	16 M and 15 L	Yes	16 M is lower than 15 L; see Fig. 4
	GL	16 M and 17 L	Yes	16 M is lower than 17 L; see Fig. 4
7	**CA**	23 S and 24 M	No	See Fig. 5
	GL	23 S and 24 M	No	See Fig. 5
	GL	23 S and 25 M	No	See Fig. 5
	GL	23 S and 28 M	No	See Fig. 5
	GL	23 S and 26 M	Yes	26 M is higher than 23 S; see Fig. 5
	GL	27 S and 24 M	No	See Fig. 5
	GL	27 S and 25 M	No	See Fig. 5
	GL	27 S and 28 M	No	See Fig. 5
	GL	27 S and 26 M	Yes	26 M is higher than 23 S and 27S; see Fig. 5
9	GL0	36 S and 37 M	No	See Fig. 6
	GL	38 S and 39 M	No	See Fig. 6
	GL	38 S and 41 M	Yes	41M is lower than 38S; see Fig. 6
	GL	40 S and 39 M	No	See Fig. 6
	GL	40 S and 41 M	Yes	41 M is lower than 40S; see Fig. 6
10	GL	42 M and 45 L	No	See Fig. 7
	GL	42 M and 47 L	No	See Fig. 7
	GL	44 M and 45 L	No	See Fig. 7
	GL	44 M and 47 L	No	See Fig. 7

In all, 24 different TM BioControl-1® samples were compared for LD_{50} variation with package size. Of these, five came from small packages (samples 23, 27, 36, 38 and 40), 13 came from medium packages (8, 10, 14, 16, 24, 25, 26, 28, 37, 39, 41, 42 and 44), and the remaining six came from large packages (9, 11, 15, 17, 45 and 47). Samples #16, #26, and #41 were the only samples showing differences in the LD_{50} values (Table 5). These three samples represent only 12.5% of the data with 21 of the 24 samples compared or 87.5% showing no differences when compared.

There were 28 different comparisons made with the 24 samples (Table 5). LD_{50} values of medium packages (samples #16 [Fig. 4] and #26 [Fig. 5]) do not agree with the LD_{50} values for other medium packages in their respective lots. Sample 16 had the lowest while sample 26 had the highest LD_{50} value among the medium size packages, i.e. they were "outliers". However, these values may not be accurate due to experimental error or individual package variation within the same package size in a lot. If the comparisons using these two samples (#16 and #26) are excluded from the overall comparisons of the effect of package sizes, then of the 28 comparisons made among package sizes, 26, (or 92.9%) show no differences in LD_{50} values (Table 5).

As stated earlier, individual TM BioControl-1® package differences could be due to single package variation. In addition, bioassays conducted over time are extremely difficult to perform in a uniform manner. There may be some experimental error such as occasional variation in the insects selected for a particular bioassay, individual differences in the insect population, or individual variation in the dose administered (although 1µl was given to each larva the number of PIBs could vary in that 1µl).

In summary, the results of the bioassays on the effects of package sizes on virus viability indicate that when comparisons are made with the same strain of Douglas-fir tussock moth, there was no difference in LD_{50} values among samples from different package sizes from the same lot. The GL-1 strain was used to bioassay 39 of the 47 stored TM BioControl-1® samples. The remaining 8 samples that were not bioassayed were samples #12 and #13 from lot 4, #18 and #19 from lot 5, #29, #30, and #31 from lot 7, and #46 from lot 10 (Table 2). Two samples from each package size had been tested and these samples would have represented a third replicate of that package size in the lot. Since there was no difference in LD_{50} values due to package size, it was felt that it was not necessary to spend additional time and resources on bioassays of the third sample or replicate. Additional bioassays would not have added anything to the already obvious results that package size did not affect the efficacy of the stored TM BioControl-1®.

3) Time in Storage

Results

Since package size did not affect the bioassay results within a lot, the bioassay data of each sample in a lot were combined and analyzed with SAS PROC PROBIT. The number of samples combined per lot is given in Table 4. This produced one LD_{50} value for each of the lots. All fresh data were combined in the same manner for an overall LD_{50} value for a fresh OpNPV sample (Table 6 and Fig. 8).

There is no significant difference (overlap of 95% fiducial limits) in the LD_{50} values for eight lots (lots 2, 3, 4, 5, 6, 7, 9, and 10). The LD_{50} for lot 1 is significantly higher ($p<0.05$) than all lots except for lot 3 and is significantly lower ($p<0.05$) than lot 8. The LD_{50} for lot 8 is significantly higher ($p<0.05$) than all other lots. These results indicate that there is a loss of potency of TM BioControl-1® over time, but it does not appear to be clearly directly related to the time in storage (Table 6).

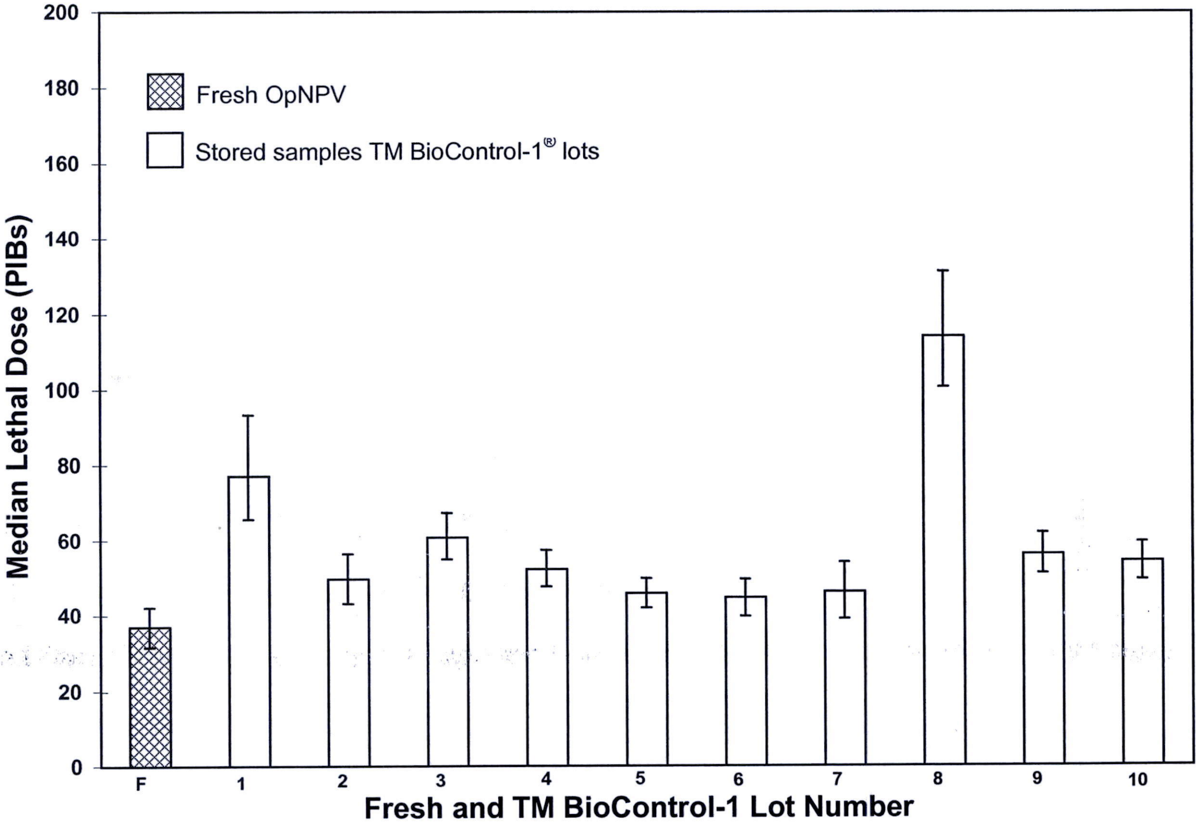
Table 6. Comparison of LD_{50} values, and potency ratios to fresh OpNPV, of the different lots of TM BioControl-1® samples stored for various lengths of time at -10°C[a]

Sample	Years in storage (to 2000)	LD_{50} (PIBs)	Potency ratio[b]
Lot 1	15	77.1	0.5
Lot 2	14	49.6	0.7
Lot 3	14	60.7	0.6
Lot 4	14	52.1	0.7
Lot 5	14	45.7	0.8
Lot 6	11	44.5	0.8
Lot 7	10	46.1	0.8
Lot 8	9	113.8	0.3
Lot 9	7	55.9	0.7
Lot 10	5	54.0	0.7
Fresh OpNPV	0	36.9	1.0

[a] Only data from bioassays done with the Goose Lake strain were used to calculate potency ratios.

[b] Potency ratio is the ratio of the LD_{50} value for the fresh sample compared to the LD_{50} value of the TM BioControl-1® samples of a lot.

Fig. 8. Comparison of the median lethal dose (LD_{50}) values, in PIBs per third instar Douglas-fir tussock moth larvae, for all samples of stored TM BioControl-1® of each lot as well as a combined fresh OpNPV LD_{50} value (F).



Potency Ratios. Another way to analyze the data is to calculate the potency ratios. The relative potency provides a convenient comparison of the differences among samples. The relative potency of two stimuli is defined as the ratio of equally effective doses (Finney 1952). Potency ratios were calculated, for bioassays in which we used the Goose Lake strain, by comparing the overall LD_{50} of a fresh OpNPV sample to the overall lot LD_{50} for each of the 10 lots of the stored TM BioControl-1® samples. Results indicate that each of the 10 lots is less potent than a fresh sample of OpNPV (Table 6 and Fig. 8). However, seven of the 10 lots have potency ratios of 0.7 or 0.8 (Table 6). Three of the lots (lots 1, 3 and 8) have the lowest potency ratios (0.5, 0.6 and 0.3, respectively). These lots have been in storage for 15, 14 and 9 years, respectively. Comparisons of the relative potency ratios also showed that there is a loss of potency with time in storage, but again this did not appear to be directly related to the time in storage. Lot 8, which had been in storage for 9 years, had the lowest potency ratio (0.3), followed by lot 1 (0.5) which had been in storage for 15 years (0.5) (Table 6).

It should be noted, however, that besides lot 3 (with a potency ratio of 0.5), lots 2, 4 and 5 have also been in storage for 14 years and their potency ratios were 0.7, 0.7 and 0.8, respectively. The low potency ratio (0.3) for lot 8 could be an anomaly.

Discussion

These results suggest that the LD_{50} values of the lots show a decrease in efficacy of the stored product, but this did not appear to be directly correlated with the length of time in storage. If length of storage had directly affected the efficacy in a linear fashion, one would have expected an increase in the LD_{50} values with increased time in storage. This was not the case, but it should be noted that at the time the bioassays were conducted, all lots had been in storage for 5 years or more. Processing the virus infected dead larvae for the product by the three companies could have additional effects that may cloud the effects that time in storage had on the efficacy of the stored product. These are only speculations based on visual impressions of the coarseness of the lots of the product and whether additional grinding was necessary to extract viral DNA from the various lots, and that the number of PIBs/g in the 10 lots were not the same (see Section 5 for details). When LD_{50} values are compared to the LD_{50} value for a fresh sample of OpNPV, there is no overlap of the 95% fiducial limits between the fresh virus and any of the 10 lots. All 10 lots have significantly higher LD_{50} values ($p<0.05$) than the fresh OpNPV. These results indicate that there was a loss of efficacy of TM BioControl-1® in storage for all lots when compared to a fresh OpNPV sample. Potency ratios also indicate that all lots are less potent now (after 5 to 15 years in storage) than a fresh sample of OpNPV. Eight of the lots have retained at least a potency of 0.7, suggesting that they have maintained most of their efficacy compared to a fresh OpNPV sample.

Three of these lots (lots 5, 6, and 7) have potency ratios of 0.8 when compared to fresh OpNPV, but this difference from the fresh OpNPV was not significant. Lot 1 and lot 8 had the largest reduction in efficacy. However, this decrease in efficacy does not appear to be directly related to time in storage since lot 8 has only been stored for 9 years, yet had the greatest loss in efficacy (0.3), and lot 1 with the longest time in storage had the second lowest (0.5) loss of efficacy.

It should be noted, however, that when the TM BioControl-1® was first produced, efficacy tests with a fresh batch of OpNPV were not performed prior to storage, and only an advance sample from each lot was bioassayed. At that time the efficacy of the lots were not compared to a fresh sample of OpNPV. It is possible that the original lots were not as effective as a fresh sample of OpNPV even before the lots were vacuum-packed for storage. Our comparisons only reports the efficacy as compared to a fresh sample and do not necessarily prove that the decrease in efficacy of the different lots, i.e. lost potency from their original state, is all due to the storage, although it is very likely and expected that this happened. What is not clear is whether loss of potency is directly related to length of storage. Loss of potency of virus over time has been reported by other researchers (Cunningham 1970, Martignoni 1978, Lewis and Rollinson 1978, Kaupp and Ebling 1993). The lower potency ratios for some of the lots may have been partly due to processing by the different organizations or companies. Generally, there is a "learning curve" for most activities and producing viral infected insects and their processing into a powder is not an exception. Lot 1 was the first lot produced and processed, and was the "coarsest" (for details see "Physical Properties of TM BioControl-1®" in Section 5 of this report).

During bioassaying of the advance samples at Corvallis, lot 6 was shown to be contaminated with CPV (see attached copy of the memo written by Paul J. Iwai). Our DNA analysis of samples of stored product (see Section 5) have also shown that, in addition to lot 6, lots 4 and 5 were also contaminated with this virus. There is a synergistic effect between these two, NPV and CPV, viruses (Tanada 1956) and this synergistic effect may explain the relatively high potency ratio (0.7, 0.8 and 0.8) for lots 4, 5 and 6, respectively. This may explain the lowering of the LD_{50} value for the lots contaminated with CPV (lots 4, 5, and 6). In addition to this, another confounding factor may be the coarseness of the various lots. The possibility of a reduction in LD_{50} values in lots contaminated with CPV could be checked in the future, should it be required, by comparing the LD_{50} values of CPV-contaminated samples against those that are not contaminated (4b versus 4a, 5b versus 5a). However, should this be the case, it does not explain why lot 7, which is free of CPV contamination, had a potency ratio as high as lots 5 and 6 (0.8), which were contaminated with CPV. (To investigate this possibility was not part of the cooperative agreement and we found out about CPV contamination during the work described in Section 5). Some of these confounding factors could have contributed to clouding the correlation between length of time in storage and loss of potency of the virus in TM BioControl-1®.

Other studies with the same stored tussock moth virus show similar loss of efficacy with storage. Martignoni (1978), using the diet surface contamination technique, reported a shelf-life of 5 years for OpNPV when the virus was stored in a cool, dry place (the exact temperature was not specified). However, it should be noted that the virus samples used in this cooperative project were stored at -10°C. Similarly, Kaupp and Ebling (1993), using a diet plug inoculation bioassay with second instar of the closely related whitemarked tussock moth (*Orgyia leucostigma* (J.E. Smith)) larvae and Virtuss® (the same virus as OpNPV, but produced in whitemarked tussock moth), have also concluded that potency of the virus decreased in storage at 4°C. A 46% loss in infectivity was observed after 2 years in storage at 4 °C and Virtuss® stored up to 10 years at 4 °C at showed up to a 25-fold decrease in infectivity.

This decreasing potency with storage was also reported by other authors with different insects. Cunningham (1970), using balsam fir (*Abies balsamea* [L.] Mill.) foliage dipped in virus suspensions, found that eastern hemlock looper (*Lambdina fiscellaria fiscellaria* (Guenee)) NPV, stored for 6 years at 4°C, showed a 200-fold loss of pathogenicity to third instar larvae compared to freshly produced virus. Lewis and Rollinson (1978) found a similar decrease with stored gypsy moth (*L. dispar* (L.)) NPV. These authors reported that suspensions of NPV retained their potency for 5 years under refrigeration, for 2 years at room temperature, 1 year as air-dried powder stored at 4°C, and 6 months as air-dried powder stored at 38°C, in diet contamination bioassays using second instar gypsy moth larvae.

Neilson and Elgee (1960), investigating the effect of storage on virulence of NPV on second and third instar larvae of European spruce sawfly (*Gilipina hercyniae* [Hartig]), reported similar results. Using foliage contaminated with virus suspension, they reported that when virus was stored at 4.5°C, loss of potency occurred after 5 years with the greatest change in virulence at 9 years in storage and total inactivation after 12 years of storage. All of the above studies reported some loss in activity of the virus with storage. Our results also indicate that there has been a loss in potency but this loss does not appear to be **directly** related to the length of time in storage at -10°C. Because TM BioControl-1® was stored at much lower temperature (-10ºC) than those reported above we would expect lower loss of potency in our samples. Perhaps storing at even lower temperature (-20°C) may further reduce the rate of loss of the efficacy of stored virus products.

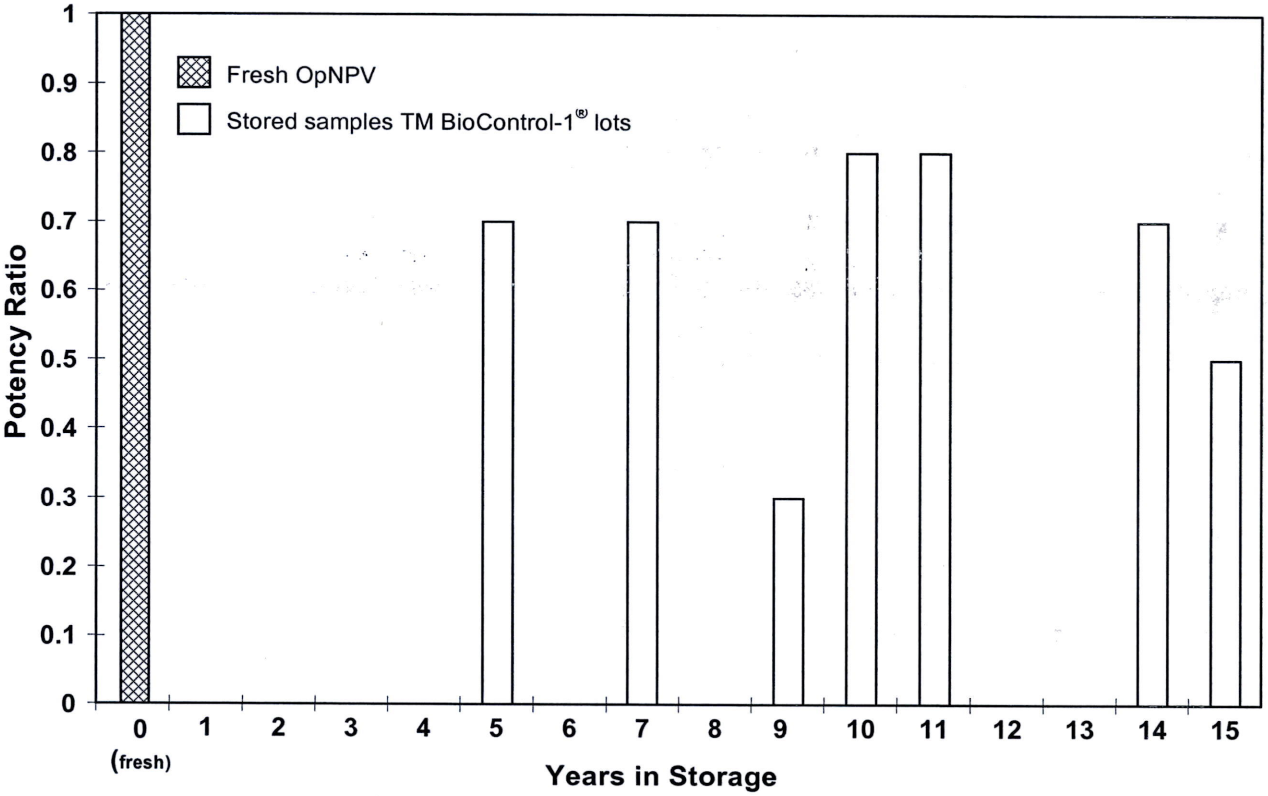
4) Differences in LD_{50} and Physical Properties of TM BioControl-1® Due to Company Processing the Virus Killed Insects

Results

The first lot (lot 1) produced, was processed by the USDA Agricultural Research Services. Three private companies were contracted to process the virus-killed Douglas-fir tussock moth larvae in the other lots. Lots 2-5 were processed by Reuter, lots 6-7 by Espro and lots 8-10 by Crop Genetics. The LD_{50} data and the potency ratios (Table 6, Fig. 8 and 9) show no obvious differences in LD_{50} values of the samples from the various lots among the four companies processing the virus-killed larvae. Lot 1 had a high LD_{50} value (77.1 PIBs) and a potency ratio of

0.5 while lot 8 had the highest LD_{50} value (113.8 PIBs) and a potency ratio of 0.3. Lot 3 also had a relatively high LD_{50} value (60.7 PIBs) and a potency ratio of 0.6. The other seven lots were all in the same range of potency ratio values (0.7-0.8). The differences in the physical properties of the product in the different lots may be partly due to the companies doing the processing, or more likely is a reflection of the learning curve and gain in experience over time by the processing companies.

Fig. 9. Comparison of potency ratios calculated for each lot of stored TM BioControl-1® against time in storage.[a]



[a] Lots 2, 3, 4, and 5 were all stored for 14 years and are combined in this graph (i.e. the potency ratio for 14 years in storage is the average potency ratio calculated from the ratios of lots 2, 3, 4 and 5).

During viral DNA extraction from the various lots of TM BioControl-1® (see Section 5), it was noticed that the lots were not the same in terms of their physical characteristics. Lot 1, produced by the USDA Agricultural Services, was very coarse. It resembled a rough mixture of large insect debris and a coarse homogenized powdered component. This lot was so coarse that grinding with a mortar and pestle was necessary to facilitate adequate sedimentation to isolate the OpNPV PIBs and extract the viral DNA. The coarseness of the stored product may, in part, explain the low (0.5) potency ratio of this lot.

Lots produced by Reuter Labs, Inc. (lots 2-5) were a more homogenous mixture. Large insect debris was not readily seen and the powdered component had a finer texture than in lot 1. Samples from lots 2-5 did not require additional grinding with mortar and pestle to facilitate extraction of the viral PIBs, although additional grinding did aid in the recovery of PIBs.
Lots 6-10, processed by Espro / Crop Genetics Inc., had the finest texture of all the lots. The texture among these lots was more consistent than the lots produced by either Reuter Labs or USDA Agricultural Services. These lots were finely ground and insect debris was not readily observable. These lots required no extra grinding to aid in PIB recovery.

During the process of extracting viral DNA from the various samples of TM BioControl-1®, it was observed that the concentration of DNA varied, sometimes substantially, even though a consistent amount of product was weighted out at the start. The recovery of DNA is not an effective way to quantify viral concentrations in samples of TM BioConrtrol-1 due to losses of viral DNA during the extraction and purification process. However, the differences in DNA recovered from lot to lot are, in some cases, larger than what could be accounted for as experimental loss. This suggests that the quantity of PIBs, and ultimately virions in the product processed by the various companies, is not uniform from lot to lot. This may explain, or at least contribute to explaining, the confounding effects of the various factors that caused loss of efficacy (degradation) of the stored product to not appear directly related to the length of time in storage.

Discussion

Lot 1 may only have a high LD_{50} value and a low potency, of 0.5, because it was the first lot produced and contained a larger amount of insect debris. Also, there could have been some initial problems in working out the protocols for the treatment of the larvae and cleaning up the dead insect samples after homogenization. There is a learning curve with developing and perfecting all new products. An examination of this lot indicated that it contained more debris than other lots, which may have resulted from cruder processing. This could also have affected the shelf life.

However, there seems to be no obvious reason why lot 8, stored for only 9 years, faired so badly. It was the first lot processed by Crop Genetics, so this may have had an effect. The first lots or production by a new agency usually have problem(s), and it may take a while to "work out the bugs". The Government contracts with the processors set out standards that had to be met and we can only assume that this was done. Not enough is known about the handling of the frozen dead larvae and the processing to reach a definite conclusion concerning how the different processors and their processing techniques affected the final efficacy of the TM BioControl-1® lots.

References Cited

Aratake, Y. 1973. Strain differences of the silkworm, *Bombyx mori* L., in the resistance to a nuclear polyhedrosis virus. J. Seric. Sci. Jpn. 42: 230-238.

Cunningham, J. C. 1970. The effect of storage on the nuclear polyhedrosis virus of the eastern Hemlock looper, *Lambdina fiscellaria fisicellaria* (Lepidoptera: Geometridae). J. Invertebr. Pathol. 16: 352-356.

Finney, D. J. 1952. Probit analysis: a statistical treatment of the sigmoid response curve, 2nd ed. Cambridge University Press, London.

Hadfield, J. S., and R. W. Magelssen. 1995. TM BioControl-1® production history and inventory status. USDA Forest Service Region 6 Bulletin, Corvallis, OR. 20 p.

Kalmakoff, J. 1980. Viruses, pp. 21-57. *In* J. Kalmakoff and J. Longworth (eds), Microbial Control of Insect Pests. Bulletin 228, New Zealand Dept. of Scientific and Industrial Research, Wellington.

Kaupp, W. J., and P. M. Ebling. 1990. Response of third, fifth, and sixth instar spruce budworm, *Choristoneura fumiferana* (Clem.), larvae to nuclear polyhedrosis virus. Can. Entomol. 122: 1037-1038.

Kaupp, W. J., and P. M. Ebling. 1993. Effect of mechanical processing and long-term storage on biological activity of Virtuss. Can. Entomol. 125: 975-977.

Lewis, F. B., and W. D. Rollinson. 1978. Effect of storage on the virulence of gypsy moth nucleopolyhedrosis inclusion bodies. J. Econ. Entomol. 71: 719-722.

Martignoni, M. E. 1978. Virus in biological control: production, activity, and safety, pp. 140-147. *In* M. H. Brookes, R. W. Stark, and R. W. Campbell (eds.), The Douglas-fir tussock moth: a synthesis. USDA Technical Bulletin 1585, USDA Forest Service, Science and Education Agency, Washington, DC.

Martignoni, M. E. 1999. History of TM BioControl-1®: the first registered virus-based product for control of a forest insect. Am. Entomol. 45: 30-37.

Martignoni, M. E., and P. J. Iwai. 1977. Peroral bioassay of technical-grade preparations of the Douglas-fir tussock moth nucleopolyhedrosis virus (Baculovirus). USDA Forest Service Research Paper PNW-222: 1-12.

Martignoni, M. E., and P. J. Iwai. 1978. Activity Standardization of technical preparations of Douglas-fir tussock moth (*Orgyia pseudosugata*) Baculovirus. J. Econ. Entomol. 71: 473-476.

Milks, M. 1997. The comparative biology and susceptibility of *Tricholpusia ni* (Lepidoptera: Noctuidae) lines to a nuclear polyhedrosis virus. Environ. Entomol. 26: 839-848.

Neilson, M. M., and D. E. Elgee. 1960. The effect of storage on the virulence of a polyhedrosis virus. J. Insect Pathol. 2: 165-171.

Otvos, I. S., J. C. Cunningham, and L. M. Friskie. 1987. Aerial application of nuclear polyhedrosis virus against Douglas-fir tussock moth, *Orygia pseudosugata* (McDunnough) (Lepidoptera: Lymantriidae): I, Impact in the year of application. Can. Entomol. 119: 697-706.

Otvos, I. S., J. C. Cunningham, L. MacLauchlan, P. Hall, and N. Conder 1998. The development and operational use of a management system for control of Douglas-fir tussock moth, *Orgyia pseudosugata* (Lepidoptera: Lymantriidae), populations at pre-outbreak levels, pp. 143-154. *In*: M. L. McManus and A. M. Liebold (eds.), Proceedings: Population Dynamics, Impacts and Integrated Management of Forest Defoliating Insects, 18-23 August 1996, Banska Stiavnica, Slovak Republic. USDA Forest Service Northeastern Research Station General Technical Report NE-247, Radnor, PA.

Reichelderfer, C. F., and C. V. Benton. 1974. Some genetic aspects of the resistance of *Spodoptera frugiperda* to a nuclear polyhedrosis virus. J. Invertebr. Pathol. 23: 378-382.

Robertson, J. L., and H. K. Preisler. 1992. Pesticide Bioassays with Arthropods. CRC Press, Boca Raton, FL.

SAS Institute. 1989-1996. SAS users guide: statistics, version 6.12. SAS Institute, Carey, NC.

Scott, D. W. 2000. Activity standardization and determination of acre dose of TM BioControl-1® preparation. Internal USDA Forest Service document.

Skatulla, V. U. 1987. On the susceptibility of *Lymantria dispar* larvae of different geographical regions to an NPV-virus. Anz. Schadlingskd. Pflanz. Umweltschutz 60: 15-18.

Tanada, Y. 1956. Some factors affecting the susceptibility of the armyworm to virus infections. J. Econ. Entomol., 49: 52-57.

Thompson, C. G., and L. J. Peterson. 1978. Rearing the Douglas-fir tussock moth. USDA Agriculture Handbook No. 520, Combined Forestry Pest Research and Development Program, Washington, DC. 20p.

United States Department of Agriculture
Forest Service
Pacific Northwest Research Station
3200 SW Jefferson Way
Corvallis, Oregon
97331

ARCHIVE.

Reply to: 4500

Date: December 1, 1988

Subject: TM BioControl-1; Assay of BM-50

To: Anita Hutchins, FPM, R-6

Bioassay (of BM-50, TM Biocontrol-1) 681 has been completed recently. This is an assay of a representative sample received on November 16 from ESPRO, Inc. All pertinent information dealing with this assay is enclosed.

Points of interest:

1. Because ESPRO uses a different processing technique, the LC_{50} in ng is about three times less than previous processed material. This is also reflected in the acre dose.

2. The LC_{50} in PIB's is the lowest (best) we have, to date, on TM BioControl-1. This could be a reflection of the processing technique.

3. There is a case of cytoplasmic polyhedrosis (see assay worksheet Group No. 10, day 6).

    a) Diagnosis confirmed by passage to four other larvae. Using diet surface contamination technique, the contaminated diet was offered to four 4th-instar larvae. The larvae were sacrificed on day 7 and all four larvae were found to be infected with cytoplasmic polyhedrosis.

    b) Also, all living test larvae were sacrificed and checked for cytoplasmic polyhedrosis infection. No cytoplasmic polyhedrosis was found in exposed or control groups.

4. All statistics with computer-run are okay.

Paul J. Iwai
PAUL J. IWAI
Biological Laboratory Technician

Enclosure

cc:
G. Larson
A. Adamson
D. Kolodny

Section 2

Testing Potential Anti-feedant Properties of the Carrier 038a® on the Douglas-fir Tussock Moth from Oregon

B. Kukan, I. S. Otvos, and A. Van de Raadt

Canadian Forest Service, Pacific Forestry Centre, Victoria, BC.

This bioassay was conducted in the spring of 2000 at the request of the USDA Forest Service to test the potential anti-feedant effect of the blank carrier 038a® that the US Forest Service was going to use in their control operation against the Douglas-fir tussock moth in Oregon in 2000. We conducted this extra bioassay, prior to the suppression activity in the field, to help plan the use of TM BioControl-1® in Oregon and to assist with the possible explanation of the results.

Introduction

The commercially produced carrier 038® (Valent BioSciences™, N. Chicago, IL) has been used successfully with the nucleopolyhedrovirus product Gypchek® (USDA Forest Service, Washington DC) to treat outbreaks of the gypsy moth, *Lymantria dispar* (L.), in the eastern United States. The purpose of the current study was to test the efficacy of the nucleopolyhedrovirus product TM BioControl-1® (USDA Forest Service, Washington DC) when combined with the carrier 038a® in the laboratory before it was incorporated into the planned Douglas-fir tussock moth suppression project in Oregon in 2000, and to investigate if carrier 038a® had any anti-feedant activity on Douglas-fir tussock moth larvae. Carrier 038® or 038a® had never been used before 2000, i.e. prior to our test in the laboratory in April, in delivering TM Biocontrol-1® to control Douglas-fir tussock moth outbreaks. The difference between carrier 038® and 038a® is that in the former lignosulfate (an additive that provides protection of the virus from UV radiation) is mixed from powdered form, while in carrier 038a® lignosulfate is added in liquid form to facilitate mixing and reduce sedimentation of lignosulfate.

Materials and Methods

Douglas-fir tussock moth egg masses, collected in Oregon in fall 1999, were shipped to Victoria, British Columbia, and there stored at 5±1°C for 4 months to break diapause. A sub-sample of the eggs (about 40-50) from each egg mass was used to determine percent parasitism, natural viral incidence and larval hatching success. The remaining eggs from all egg masses were used in an efficacy trial of the stored product delivered in carrier 038a®. These remaining eggs were allowed to hatch and larvae reared at 25±1°C, 50-60% RH with a photo-period of 16:8 (L:D). Larvae were reared in groups of 10 on artificial diet (Thompson and Peterson 1978) in petri dishes until they reached second instar when force-flushed new shoots of Douglas-fir (*Pseudotsuga menziesii* [Mirbel] Franco), decontaminated in 5% bleach, were added to the dishes to "acclimatize" the larvae to feeding on foliage.

Virus lot 4 was selected for use in the Douglas-fir tussock moth suppression program in Oregon in 2000. TM BioControl-1® sample 9 (1840 g/pkg) from lot 4 was selected for testing since it had the lowest potency and consequently required the highest g/acre-dose treatment of the samples tested, from lots 2-6, up to that point. The g/acre-dose, calculated from sample LD_{50} for the purpose of this experiment, was 5.1 g/acre. Carrier 038a® for the test was provided to us by Dr. Richard Reardon, USDA Forest Service, Morgantown, WV.

The experiment with the blank carrier (038a®) was done in April 2000, in an attempt to provide data for the suppression project on the potential anti-feeding effect of the carrier. New shoots, on which second and third instar larvae feed, were not yet available in the field. For this reason, we developed a scaled-down version of the field application rate of 1 gal/acre that we could apply in the laboratory to a relatively small area (1 square ft) of new foliage and extrapolate from the results of this small scale experiment back to the application rate in the field.

Twenty 2-year old Douglas-fir seedlings were force-flushed in the greenhouse, and the new shoots clipped and arranged to cover a 1 square ft area in a tight but not overlapping fashion. The new shoots were taped with double-sided tape to the center of a 50 cm x 50 cm sheet of foam-core board covered with brown paper that was replaced after each treatment with a new one. Treated foliage was allowed to dry for 30 min following treatment application and then placed in a plastic bag and stored at 4°C until needed. It was assumed that the 1 square ft area of new shoots and its treatment in the laboratory would represent 1 square ft of 1 acre of foliage with the appropriate treatments in the field.

There were four treatments (three applied to force-flushed new shoots and one to diet) and three controls. Each of these seven 'treatments' was replicated 10 times. Each of these 'treatments' consisted of five newly-molted third instar larvae placed into a clean petri dish (15 x 100 mm) with fresh foliage or a piece of diet treated with the virus applied in water or carrier 038a®. In the case of the controls the foliage was treated with the blank, undiluted carrier 038a®, or distilled water.

The insects were allowed to feed on the treated foliage and were monitored after 24, 48, and 120 hours. From previous, other preliminary, experiments we found that approximately 2.5 cm pieces of new foliage will last about 5 days in plastic cups (Solo P100, Solo Cup Co., Urbana, IL 61801-2895) before they dry out under the rearing conditions we used (25±1°C, 50-60% RH, 16:8 [L:D]). This happens to be the maximum half-life of the virus under natural conditions in the field. Feeding was scored by observing and estimating the amount of frass that had accumulated in the bottom of the petri dish and how many larvae were actively feeding at the time of observation. In addition, at 120 hours an estimate was made of the amount of foliage that had been consumed. At 120 hours the new foliage had dried out and was replaced in all dishes with fresh Douglas-fir twigs that had been decontaminated but left untreated. Insect mortality was recorded daily for 15 days. NPV infection was determined by gross pathology and confirmed under compound microscope at 400X magnification.

Treatments

Controls.

1) Distilled water at the equivalent rate of 1 gal/acre on foliage
2) Undiluted carrier 038a® at the equivalent rate of 1 gal/acre on foliage
3) Undiluted carrier 038a® at 2.5X the recommended equivalent rate of 1 gal/acre on foliage

TM BioControl-1® Treatments. With the exception of treatment number 4 on diet, virus was sprayed onto clipped "force-flushed" new foliage of 2 year-old Douglas-fir seedlings.

1) TM BioControl-1® at 5.1 g/acre equivalent dose in distilled water at the equivalent rate of 1gal/acre on foliage
2) TM BioControl-1® at 5.1 g/acre equivalent dose in carrier 038a® at the equivalent rate of 1 gal/acre on foliage
3) TM BioControl-1® at 2.97 g/acre equivalent dose in carrier 038a® at the equivalent rate of 1 gal/acre on foliage
4) TM BioControl-1® at 5.1g/acre equivalent dose in carrier 038a® at the equivalent rate of 1 gal/acre smeared on the surface of a diet cube (2.5 cm x 2.5 cm x 1 cm)

Results and Discussion

Feeding Behavior

Table 1 shows frass accumulation (estimated as percent of the bottom of the petri dish covered) and estimates of the remaining foliage. Both of these are subjective observations (not quantitative measurements), and are only meant to give an idea of the overall feeding taking place. Observations of active feeding are quantitative and represent only two points (24 and 48 h) in time. Data for frass and active feeding observations for 120 h are not included in Table 1 since larvae were preparing to molt and feeding had slowed down considerably. Frass accumulation indicated that generally more feeding occurred in the controls than in dishes containing virus treated foliage or diet. The number of larvae actively feeding was generally higher in the controls, especially in those having carrier 038a® (controls 2 and 3), than in the treatments where the foliage (treatments 1-3) and diet (treatment 4) were also treated with TM BioControl-1® (Table 1).

Generally, there was less feeding by the larvae observed on the foliage that had been treated with TM BioControl-1® than those in the controls, where the foliage received distilled water or carrier 038a®. Interestingly, there seemed to be more feeding on the virus + 038a® (the carrier) treated foliage in the petri dishes (treatment 2) than in the dishes with virus only in distilled water treatment (treatment 1), or in treatment 3 where only 2.97 g equivalent of virus was applied versus 5.1 g equivalent of virus applied in treatment 2, or in treatment 4 where the virus was applied in carrier 038a® to artificial diet. These results suggest that the virus in TM BioControl-1® may have some anti-feedant properties similar to other viruses (Kukan unpublished data). The blank, undiluted carrier 038a® did not appear to have any anti-feedant properties and may even act as a “feeding stimulant” even when it is combined with virus in the TM BioControl-1® product (Table 1).

Summary of Feeding Behavior. Generally, more feeding occurred in the controls than in the treatments with the virus. The number of actively feeding larvae seemed to be higher in the controls including those feeding on the 038a® treated foliage than on virus-treated foliage or diet (Table 1). There was less feeding observed by the larvae on the food treated with TM BioControl-1®. These results suggest that the TM BioControl-1®, like other viruses, may have some anti-feedant properties. Carrier 038a® did not appear to have any anti-feedant properties and it may even have some feeding stimulant properties even when combined with TM BioControl-1® for the control of the Douglas-fir tussock moth. That is, the “feeding stimulant” properties of carrier 038a® are greater than the “anti-feeding” properties of the virus in the TM BioControl-1® product.

Table 1. Results of the laboratory experiment testing the potential anti-feeding effects of the undiluted carrier 038a® to be used in the Douglas-fir tussock moth suppression project in Oregon in 2000[a]

Treatment[b]	% of dish covered by frass		% larvae actively feeding		% foliage remaining at 120 h on the branch tip
	24 h	48 h	24 h	48 h	
Controls					
1) Distilled water (at the equivalent rate of 1 gal/acre) on foliage	85	95	74	20	45
2) Undiluted carrier 038a® (at the equivalent rate of 1 gal/acre) on foliage	38	68	50	38	27
3) 2.5X undiluted carrier 038a® (at the equivalent rate of 2.5 gal/acre) on foliage	68	95	74	34	35
TM BioControl-1® Treatments					
1) 5.1 g/acre equivalent virus in distilled H_2O (at the equivalent rate of 1 gal/acre) on foliage	36	70	34	32	41
2) 5.1 g/acre equivalent virus in undiluted carrier 038a® (at the equivalent rate of 1 gal/acre) on foliage	21	65	52	44	44
3) 2.97 g/acre equivalent virus in undiluted carrier 038a® (at the equivalent rate of 1 gal/acre) on foliage	23	70	52	44	51
4) 5.1 g/acre equivalent virus in undiluted carrier 038a® (at the equivalent rate of 1 gal/acre) on diet	10	25	4	0	NA

---

[a] Following the successful use of carrier 038a® in Oregon in 2000 the carrier was also used successfully in 2001.

[b] All controls and treatments were applied to the 1 square ft of foliage or the diet at the volume equivalent of 1 gal of solution/acre (the intended volume of application in Oregon), except for Control 3 which was applied at a rate equivalent to 2.5 gal of carrier 038a®/acre. In the four treatments the virus dose equivalents were: 5.1 g TM BioControl-1® per acre in distilled water and carrier 038a® in treatments 1 and 2, respectively, 2.97 g/acre in carrier 038a®, and 5.1 g/acre in carrier 038a®. The first three treatments were applied to force-flushed new shoots of Douglas-fir, and the fourth to artificial diet.

Mortality Data

There was very little mortality in the controls (Table 2). One larva died on day 5 in the distilled water control (control 1) and one larva died on day 7 in the 038a® control (1 gal/acre equivalent; control 2). By day 15 control larvae had started to pupate; therefore, the experiment was terminated.

Mortality was high among the larvae feeding on food treated with TM BioControl-1® (Table 2). The highest mortality 15 days after infection was recorded among larvae feeding on the artificial diet treated with TM BioControl-1® (treatment 4 with 100%), followed by the same virus dose equivalent in carrier 038a® (treatment 2). Larvae given 5.1 g equivalent virus dose in 1 gal carrier 038a®/acre equivalent (treatment 2) had an average mortality of 96%, with 100% larval mortality having occurred in 9 of the 10 petri dishes and in the tenth dish 60% of the larvae died. In treatment 1, 5.1 g/acre equivalent virus in distilled water treatment, the average mortality for the treatment was slightly lower, averaging 88% mortality, suggesting that TM BioControl-1® had some anti-feeding properties. Seven petri dishes had 100% larval mortality, one dish had 80%, one dish had 60%, and one dish had 40% larval mortality. In the lower acre-dose treatment, 2.97 g/acre equivalent virus in 1 gal carrier 038a®/acre equivalent (treatment 3), the average mortality was 86%: four petri dishes had 100% larval mortality, five dishes had 80%, and one dish had 60% larval mortality. Mortality of larvae given the equivalent of 5.1 g virus dose in 1 gal carrier 038a®/acre equivalent (treatment 2) was higher (96%) than the mortality among the larvae given the same dose of virus in distilled water (88%) (treatment 1). This suggests that enhanced feeding had taken place in the presence of carrier 038a® resulting in a higher infection rate. Larvae feeding on foliage treated with a lower dose of TM BioControl-1® (2.97 g/acre equivalent virus in 1 gal carrier 038a®/acre equivalent) also experienced high mortality (86%) (treatment 3) suggesting that treatment at this dose will likely give acceptable population suppression in the field if spray deposit is good. These mortality rates represent death from the primary infection among the larvae and do not reflect the effects of the wave of secondary infection that would likely occur in the field if treatment were applied to third instar or younger larvae. This second wave of infection would cause additional larval mortality and would likely result in the collapse of the outbreak.

Table 2. Mortality of Douglas-fir tussock moth larvae reared from field collected egg masses in Oregon, challenged by various virus treatments in carrier 038a® in the laboratory[a]

Treatment	# larvae treated	# dead at day 15	% mortality at day 15
Controls			
1) Distilled water (at the equivalent rate of 1 gal/acre) on foliage	50	1	2
2) Undiluted carrier 038a® (at the equivalent rate of 1 gal/acre) on foliage	50	1	2
3) 2.5X undiluted carrier 038a® (at the equivalent rate of 2.5 gal/acre) on foliage	50	0	0
Treatments with TM BioControl-1®			
1) 5.1 g/acre equivalent virus in distilled H_2O (at the equivalent rate of 1 gal/acre) on foliage	50	44	88
2) 5.1 g/acre equivalent virus in undiluted carrier 038a® (at the equivalent rate of 1 gal/acre) on foliage	50	48	96
3) 2.97 g/acre equivalent virus in undiluted carrier 038a® (at the equivalent rate of 1 gal/acre) on foliage	50	43	86
4) 5.1 g/acre equivalent virus in undiluted carrier 038a® (at the equivalent rate of 1 gal/acre) on diet	50	50	100

[a] All controls and treatments were applied to the 1 square ft of forced-new foliage or the diet at the volume equivalent of 1 gal of solution/acre (the intended volume of application in Oregon), except for Control 3 which was applied at a rate equivalent to 2.5 gal of carrier 038a®/acre.

Summary of Mortality Data. The highest larval mortality 15 days after infection was recorded in larvae feeding on TM BioControl-1® treated artificial diet (100%) (treatment 4). Mortality of larvae receiving the same dose, i.e. the equivalent of 5.1 g/acre virus in the equivalent of 1 gal carrier 038a®/acre, was almost as high (96%) (treatment 2), and was higher than when larvae were feeding on foliage treated with the same dose of virus but in distilled water (88%) (treatment 1). This suggests that enhanced larval feeding had taken place when 038a® was present resulting in a higher infection rate. Feeding of larvae on foliage treated with a lower dose of TM BioControl-1® (equivalent of 2.97 g virus in 1 gal carrier 038a®/acre) also resulted in high mortality (86%) and this mortality was only slightly less than that caused by the 5.1 g/acre of virus in distilled water treatment. We included this lower dose (2.97 g/acre) in our laboratory test because we felt that the 5.1 g/acre-dose may be too high for second instar larvae (5.1 g/acre had been calculated from the Activity Standardization bioassays we did under the Cooperative Research Agreement using third instar larvae).

In summary, carrier 038a® does not have anti-feeding properties on Douglas-fir tussock moth, and, in fact, it may have some “feeding stimulant” effects. Therefore, its use as a carrier to deliver TM BioControl-1® to Douglas-fir tussock moth in suppression projects is justified.

References Cited

Thompson, C. G., and L. J. Peterson. 1978. Rearing the Douglas-fir tussock moth. USDA Agriculture Handbook No. 520, Combined Forestry Pest Research and Development Program, Washington, DC. 20pp.

Section 3

Comparison of Laboratory Response of Second-instar and Third-instar Larvae of Douglas-fir Tussock Moth to TM BioControl-1® Using the Diet Surface Contamination and the Diet Plug Methods

B. Kukan[1] and I.S. Otvos[1]

[1] Natural Resources Canada, Canadian Forest Service, Pacific Forestry
Centre, Victoria, BC, V8Z 1M5, Canada.

Introduction

After the discovery and the determination of the role of Douglas-fir tussock moth nucleopolyhedrovirus (OpNPV) in the population dynamics of the Douglas-fir tussock moth (*Orgyia pseudotsugata* [McDunnough]), the USDA Forest Service produced, registered and stockpiled quantities of this virus for future use (Martignoni 1978, 1999, Hadfield and Magelssen 1995). Potency of insecticidal products are measured by response of insects in bioassays. These bioassays are constantly improved to make them more accurate.

The original bioassay work, done at the time of TM BioControl-1® production on the samples provided, and prior to storage, employed the diet surface contamination technique with second instar Douglas-fir tussock moth larvae of the Goose Lake colony (Martignoni 1978, 1999). In our study of determining efficacy of stored TM BioControl-1® product, we used the diet plug inoculation technique, developed later (Kaupp and Ebling 1990), with third instar larvae of the same strain reared individually. Diet plug bioassay, along with individual rearing of infected larvae, is superior to diet surface contamination bioassay because it eliminates the variation in the distribution of polyhedral inclusion bodies (PIBs) on the diet surface, negates differences in larval feeding rates, insures that all larvae in the bioassays consume the same known quantity of virus, and avoids interactions among the group of larvae reared in the same container. That is, by using the diet plug method the exact dose consumed by each larva is known and an accurate median lethal dose (LD_{50}) can be calculated.

To resolve the differences between the response of second and third instar larvae to the virus in the two different bioassays, we undertook a comparative study in the laboratory in which we compared the responses of these two instars exposed to OpNPV, the nucleopolyhedrovirus part of TM BioControl-1®, by the two methods.

Our activity standardization studies are only one part of the process needed for proper acre-dose determination of the product (Martignoni 1978, Scott 2000). Ideally, two bioassays need to be done with each population slated for treatment when acre-dose values are determined in the ideal and correct way. The first one is a preliminary or "range-finding" bioassay to determine the response of the population and to select appropriate concentrations of virus for the second bioassay. The second bioassay is needed to accurately determine the LD_{50} values that can be used to calculate the acre-dose value of the product to be used per acre in a suppression project. Larvae for the second bioassay must come from the field population(s) that will be treated. Sensitivity of field populations of Douglas-fir tussock moth to infection by OpNPV is known to vary with geographic location (see Section 1 for details). Acre-dose determination is the second step in preparation for application, and should be done each time a new field population is scheduled for treatment or a different lot of the stored TM BioControl-1® is used for suppression.

We did this comparative study hoping that the data obtained from our experiment (comparing the diet surface contamination with the more accurate diet plug inoculation and comparing the responses of second instar versus third instar larvae) may help forest managers to calculate acre-dose in the future more expeditiously since it may not always be possible in every suppression project to run the two bioassays with the target field populations of Douglas-fir tussock moth.

Materials and Methods

Bioassay Protocol

The Douglas-fir tussock moth larvae used in the experiment were from the Goose Lake colony (GL-1 strain), maintained at the Pacific Forestry Centre since March 1996. To reduce potential virus contamination, the egg masses were gently teased apart and the eggs were washed three times with a 0.1% sodium hypochlorite (2% bleach) solution, rinsed three times with distilled water and allowed to air dry. The eggs and subsequent larvae were reared at 25±1°C, 50-60% RH with a photoperiod of 16:8 (L:D). Larvae were reared in groups of 10 on artificial diet (Thompson and Peterson 1978) in petri dishes. Newly molted second and then newly molted third instar larvae were selected from the same colony and starved for 16-20 h before the bioassays.

Virus Preparation

Twenty mg of each of the stored TM BioControl-1® samples to be tested was weighed out and mixed with 20 ml of distilled water. The viral solutions were mixed by magnetic stirrer for 2 h and viral solutions were prepared in duplicate for the experiments with second instar larvae and in triplicate for the third instar larvae. One week prior to inoculation, serial dilutions of the virus were made and stored at 4°C until inoculation (Table 1). The USDA Forest Service had counted the PIB concentrations of the TM BioControl-1® samples using the haemocytometer method (Kalmakoff and Longworth 1980) and these counts were available in the original bioassay data provided by Roy Magelssen (Hadfield and Magelssen 1995). However, as a check, haemocytometer counts of PIBs were done on five randomly selected TM BioControl-1® samples (sample #2 from lot 2, sample #20 from lot 6, samples #36 and #37 from lot 9 and sample #43 from lot 10). The current PIBs/ml calculated in our experiment were the same as the original PIB counts done at Corvallis, OR. Therefore, it was deemed unnecessary to recount the remaining samples sent for bioassaying. Serial dilutions were based on the original counts.

A fresh sample of OpNPV, to be used as a positive control, was produced prior to the bioassay. The fresh viral sample was prepared by passing OpNPV (TM BioControl-1® [sample #1 from lot 1]) once through Goose Lake strain Douglas-fir tussock moth larvae. One hundred larvae previously inoculated with OpNPV from lot 1 and reared until death, were homogenized in 5 ml of distilled water. The homogenate was filtered through cheesecloth, centrifuged once at 10 x g for 5 min and twice at 7100 x g for 20 min then resuspended in distilled water. PIB counts were done using a haemocytometer to quantify the virus. The fresh OpNPV sample was diluted based on the counts and stored at 4°C until time for the experiment. None of the serial viral dilutions were kept longer than 3 months. The virus doses of the samples used in the experiments are given in Table 1. For the diet surface experiment, virus concentrations were the same as those used in the original bioassays (Martignoni and Iwai 1977). Concentrations used in the diet plug bioassays were determined by considering results from previous bioassay experiments done in earlier efficacy trials (Kukan et al. 2000). Distilled water was used as a negative control.

Table 1. Doses of TM BioControl-1® production lot samples used to inoculate second and third instar Douglas-fir tussock moth larvae of the Goose Lake (GL-1) strain using the diet surface and diet plug methods

Experiment number	Lot	TM sample #	Time in storage	Concentration #	Virus concentrations (PIBs/µl)		
					Diet surface 2nd 25 µl	Diet plug 2nd 1 µl	Diet plug 3rd 1 µl
1	4	8	14 yr	1		4.0	
				2		8.0	
				3		16.0	16.0
				4		32.0	32.0
				5		64.0	64.0
				6		128.0	128.0
							183.0
	7	25	10 yr	1		4.4	
				2		8.7	
				3		17.3	17.3
				4		34.5	34.5
				5		69.0	69.0
				6		138.0	138.0
							197.0
	9	39	7 yr	1		4.0	
				2		8.0	
				3		15.9	15.9
				4		31.8	31.8
				5		63.5	63.5
				6		127.0	127.0
							181.0
	Fresh sample		0 yr	1		4.2	
				2		8.4	
				3		16.7	
				4		33.4	33.4
				5		66.8	66.8
				6		133.5	133.5
							190.7
2	3	7	14 yr	1	5.8	4.0	
				2	11.6	8.1	
				3	23.1	16.1	16.1
				4	46.2	32.3	32.3
				5	92.4	64.5	64.5
							129.0
							184.3
	7	27	10 yr	1	4.6	4.4	
				2	9.1	8.7	
				3	18.2	17.3	17.3
				4	36.4	34.5	34.5
				5	72.9	69.0	69.0
							138.0
							197.0
	9	41	7 yr	1	3.4	4.0	
				2	6.9	8.0	
				3	13.7	15.9	15.9
				4	27.5	31.8	31.8
				5	54.9	63.5	63.5
							127.0
							181.4
	Fresh sample		0 yr	1	4.0	4.2	
				2	8.0	8.4	
				3	16.0	16.7	16.7
				4	32.0	33.4	33.4
				5	63.6	66.8	66.8
							133.5
							190.7

Inoculation

Diet Plug. The diet plug inoculation bioassay technique we used was similar to that of Kaupp and Ebling (1990). One µl of each viral dilution or control (distilled water) was added to a small diet plug (3-4 mg) inside each well of a 24-well tissue culture plate. Previous experiments have shown that the diet plugs were large enough to fully absorb the 1 µl of liquid, and small enough for one larva to eat in 24 h. Immediately after inoculation, one second or one third instar larva (starved for 16-20 h) was placed individually into each well to feed on the treated diet plug. Five or six virus concentrations and one control were tested for each of the TM BioControl-1® samples, as well as a fresh OpNPV sample. Bioassay of the TM BioControl-1® samples, the fresh preparation of OpNPV, and an untreated control were replicated twice for the second instar experiments and three times for the bioassays using third instar larvae. There were 24 larvae used per concentration.

Larvae were held in darkness for 24 h with the inoculated diet plug at 25±1°C, 50-60% relative humidity. Larvae that consumed the entire diet plug were placed in individual cups (Solo P100, Solo Cup Co. Urbana, Illinois 61801-2895) with a fresh cube of untreated diet and returned to the same growth chamber. Larvae that did not consume the entire diet plug were discarded. Because of the high virulence of OpNPV, it was necessary to rear the larvae individually after inoculation to avoid cross infection. Separate growth chambers, set at the same rearing conditions, were maintained for rearing the control and viral infected insects to guard against viral transmission to the control insects. Diet was changed at least weekly, or more often if the diet dried out. Larvae were reared and examined for 21 days and the mortality recorded daily. Only larvae that died from NPV infection, as determined by gross pathology and confirmed by microscopic examination at 400x, were included in the analysis.

For second instar larvae, using the diet plug method, two experiments were conducted in which six TM BioControl-1® samples and two fresh OpNPV samples were tested. Two replicates of 24 larvae each were used for each concentration of treatment. In the diet plug experiment 1 with second instar larvae (see Table 1 and 2), six concentrations of virus were tested and the samples tested were as follows: sample #8 from lot 4 stored 14 years, #25 from lot 7 stored 10 years, and #39 from lot 9 stored 7 years. In experiment 2, five concentrations of virus were tested from the following samples: #7 from lot 3 stored 14 years, #27 from lot 7 stored 10 years, and #41 from lot 9 stored 7 years. In experiment 1, 1,200 larvae were tested, and 1,008 larvae were tested in experiment 2 (Table 2).

Table 2. Number of larvae used in the diet plug bioassay testing samples of TM BioControl-1® samples stored for various lengths of time against second instar Douglas-fir tussock moth larvae of the Goose Lake (GL-1) strain

Lot number	TM sample number	Length of time in storage (yr)	Number of GL-1 larvae per Replicate A	Number of GL-1 larvae per Replicate B
Experiment 1[a]				
4a	8	14	144	144
7c	25	10	144	144
9c	39	7	144	144
Fresh		0	144	144
Control			24	24
TOTAL			600	600
Experiment 2[b]				
3	7	14	120	120
7a	27	10	120	120
9c	41	7	120	120
Fresh		0	120	120
Control			24	24
TOTAL			504	504

[a] Experiment 1
Number of larvae used per sample: 6 concentrations x 24 = 144
Number of larvae used per replicate: (4 samples x 144) + control = 576 + 24 = 600
Number of larvae used in experiment 1: 2 replicates x 600 = 1,200

[b] Experiment 2
Number of larvae used per sample: 5 concentrations x 24 = 120
Number of larvae used per replicate: (4 samples x 120) + control = 480 + 24 = 504
Number of larvae used in experiment 2: 2 replicates x 508 = 1,008

For third instars, two experiments were also conducted, with the same TM BioControl-1® samples as were tested with second instar larvae. In these experiments, three replicates were used and 48 larvae per viral concentration (Tables 1 and 3). Five virus concentrations were tested for a total of 3,024 larvae per experiment.

Table 3. Number of larvae used in the diet plug bioassays testing samples of TM BioControl-1® stored for various lengths of time against third instar Douglas-fir tussock moth larvae of the Goose Lake (GL-1) strain

Lot number	TM sample number	Length of time in storage (yr)	Number of GL-1 larvae per Replicate A	Number of GL-1 larvae per Replicate B	Number of GL-1 larvae per Replicate C
Experiment 1[a]					
4a	8	14	240	240	240
7c	25	10	240	240	240
9c	39	7	240	240	240
Fresh		0	240	240	240
Controls			48	48	48
TOTAL			1,008	1,008	1,008
Experiment 2[a]					
3	7	14	240	240	240
7a	27	10	240	240	240
9c	41	7	240	240	240
Fresh		0	240	240	240
Controls			48	48	48
TOTAL			1,008	1,008	1,008

[a] Number of larvae used per sample: 5 concentrations x 48 = 240
Number of larvae used per replicate: (4 samples x 240) + control = 960 + 48 = 1,008
Number of larvae used in experiment: 3 replicates x 1,008 = 3,024

Diet Surface. The diet surface contamination/inoculation experiments were conducted as described in Martignoni and Iwai (1977), and Martignoni (1978, 1999). Fresh diet was prepared (Thompson and Peterson 1978) and 1 ml of it added to each of the wells in a 24 well plate. The diet was air dried for several hours to prevent moisture accumulation on the surface. A volume of 25 μl of distilled water or virus preparation was added to the diet surface and left for 3 h to be absorbed into the diet. One freshly molted second instar larva was added to each well and monitored daily for 14 days. The rearing conditions were the same as for the diet plug experiments (25±1°C, 50-60% RH, 16L:8D). Diet was not changed in these experiments in order to give the larvae time to consume the virus-contaminated diet. Three TM BioControl-1® samples stored for 14, 10, and 7 years, respectively, and a fresh virus sample were tested (Table 1 and Table 4). The TM BioControl-1® samples tested were the same as those used in experiment 2 of second and third instar diet plug. Two replicates of 24 larvae per concentration were inoculated. In all 1,008 larvae were tested.

Table 4. Number of larvae used in the diet surface contamination bioassays testing samples of TM BioControl-1® stored for various lengths of time against second instar Douglas-fir tussock moth larvae of the Goose Lake (GL-1) strain[a]

Lot number	TM sample number	Length of time in storage (yr)	Number of GL-1 larvae per Replicate A	Number of GL-1 larvae per Replicate B
3	7	14	120	120
7a	27	10	120	120
9c	41	7	120	120
Fresh		0	120	120
Control			24	24
TOTAL			504	504

[a] Number of larvae used per sample: 5 concentrations x 24 = 120
Number of larvae used per replicate: (4 samples x 120) + control = 480 + 24 = 504
Number of larvae used in experiment: 2 replicates x 504 = 1,008

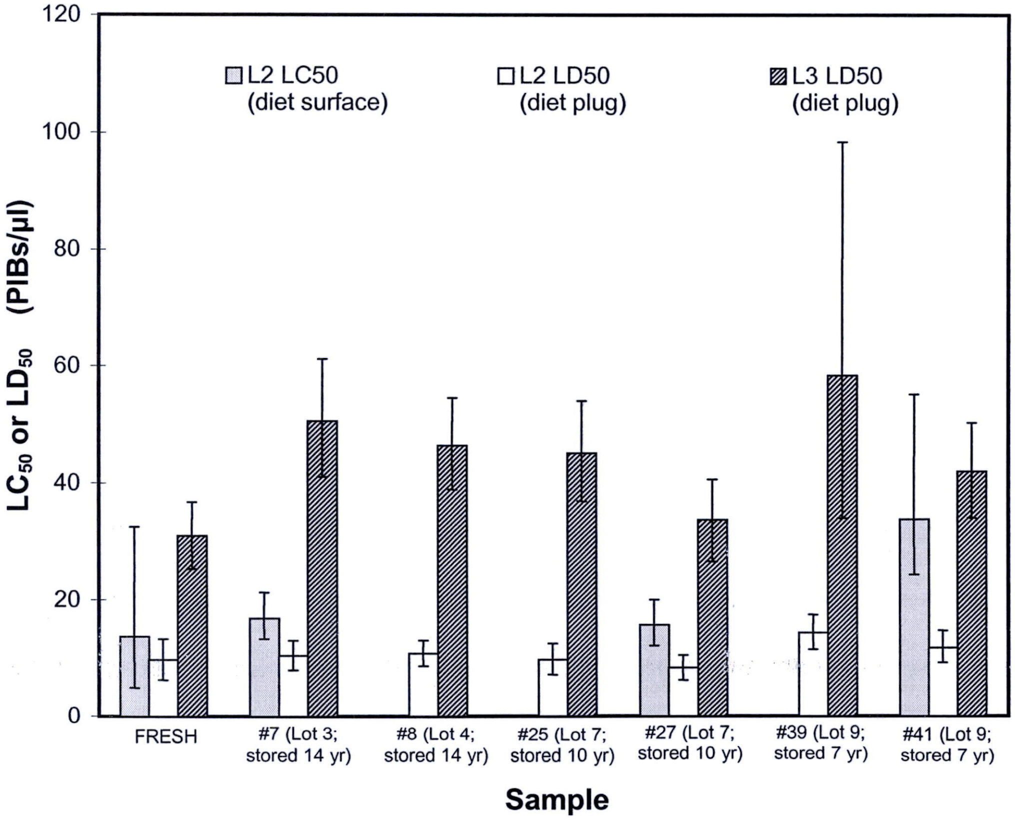
Data Analysis

Using the larval mortality data, dosage mortality curves and LD_{50} values with associated 95% fiducial limits were calculated using PROC PROBIT analysis (SAS Institute 1989-1996). Each replicate was tested as a separate preparation to verify that there were no differences in the replicates. If there was no difference, data for the replicates were combined. Estimates of medial lethal dose (LD_{50}) (diet plug) or lethal concentration (LC_{50}) (diet surface) for each TM BioControl-1® sample and the fresh OpNPV sample were calculated. LD_{50} and LC_{50} values were examined for significant differences (no overlap of the 95% fiducial limits).

Results

The doses of the TM BioControl-1® samples used to inoculate second and third instar larvae by the two methods are given in Table 1. The LC_{50} values from the diet surface contamination experiment with second instar larvae and the LD_{50} values from the diet plug experiments with the second and third instar larvae are compared in Figure 1. As expected, the LD_{50} values were significantly higher ($p<0.05$) for the third instar than for the second instar larvae in the diet plug experiment for the same virus samples in all cases, irrespective of the length of storage, even for the fresh sample. The LC_{50} values calculated for the second instars in the diet surface contamination bioassays were also significantly higher ($p<0.05$) than the LD_{50} values calculated from the diet plug bioassays (second instar) for the same TM BioControl-1® samples in all but the fresh sample. The LC_{50} values calculated for the second instars in the diet surface contamination bioassays were significantly lower ($p<0.05$) when compared to the LD_{50} values for the same samples tested with third instars for samples #7 and #27 but not for #41 or the fresh virus in the diet plug experiment.

Fig. 1. Comparison of LC_{50} and LD_{50} values from bioassays testing fresh and samples from lots of TM BioControl-1® stored for various lengths of time against second and third instar Douglas-fir tussock moth larvae (GL-1 strain).[a]



[a] Bars represent 95% fiducial limits. Numbers under the bar graphs denote sample number.

When the LD_{50} values for the third instars were compared with the LD_{50} values for the second instar larvae in the diet plug bioassays, LD_{50} values of samples for the third instars were 3.6 to 4.9 times higher than LD_{50} values of the second instars (Table 5).

When LC_{50} values calculated for diet surface contamination bioassays for the second instar larvae were compared with the LD_{50} values for the same instar in the diet plug bioassays the LC_{50} values for the diet surface experiments were 1.4 to 2.9 times higher for the samples than the LD_{50} values from the diet plug bioassays (Table 6).

Table 5. Comparison of the LD_{50} values (ratio) obtained from bioassaying samples of TM BioControl-1® stored for various lengths of time against second and third instar Douglas-fir tussock moth larvae of the Goose Lake (GL-1) strain using the diet plug inoculation technique

Lot number	TM sample number	Length of time in storage (yr)	Ratio of median lethal dose (L3 plug / L2 plug)
3	7	14	4.9
4a	8	14	4.3
7a	27	10	4.0
7c	25	10	4.6
9c	39	7	4.1
9c	41	7	3.6
Fresh		0	4.2

Table 6. Comparison of LC_{50} and LD_{50} values (ratio) obtained from bioassaying samples of TM BioControl-1® against second instar Douglas-fir tussock moth larvae of the Goose Lake (GL-1) strain using the diet surface and diet plug methods

Lot number	TM sample number	Length of time in storage (yr)	Ratio of median lethal concentration to median lethal dose (L2 surface / L2 plug)
3	7	14	1.6
7a	27	10	1.9
9c	41	7	2.9
Fresh		0	1.4

When the LD_{50} values from the third instar diet plug bioassays were compared with the LC_{50} values for the second instar diet surface contamination bioassays, the LD_{50} values of the third instars in the diet plug bioassays were 1.2 to 3.0 times higher than LC_{50} values of the second instars in the diet surface bioassays (Table 7).

When the original LC_{50} values, determined at the time of production and prior to storage, were compared to the LC_{50} values obtained in our study now with samples of the same lot, the ratio of the two LC_{50} values ranged from 0.4 to 0.7 (Table 8). In both the original and our bioassays of LC_{50} determination second instar Douglas-fir tussock moth larvae of the Goose Lake strain were used.

Table 7. Comparison (ratio) of the LD_{50} and LC_{50} values from bioassays testing TM BioControl-1® samples stored for various lengths of time against third instar larvae using the diet plug method and second instar larvae using the diet surface contamination technique[a]

Lot number	TM sample number	Length of time in storage (yr)	Ratio of median lethal dose to median lethal concentration (L3 plug / L2 surface)
3	7	14	3.0
7a	27	10	2.2
9c	41	7	1.2
Fresh sample		0	2.3

[a] LD_{50} values were obtained for third instar larvae using the diet plug method and the LC_{50} values were obtained using the diet surface contamination technique with second instar larvae of the Goose Lake strain.

Table 8. Comparison (ratio) of LC_{50} values obtained at the time of production with LC_{50} determined in our current bioassays for the same TM BioControl-1® lots after storage[a]

Lot number	TM sample number	Length of time in storage (yr)	Original LC_{50} PIBs/µl	Current LC_{50} PIBs/µl	Ratio of original LC_{50}/current LC_{50}
3	7	14	17.9	25.7	0.7
7a	27	10	15.3	21.4	0.7
9c	41	7	20.6	47.1	0.4

[a] Both in the original (at the time of production) and current bioassays, the diet surface contamination method was used on second instar larvae of the Goose Lake strain of the Douglas-fir tussock moth.

We have also compared the LC_{50} values "original" and "current" in a different way (Table 9). The "current" value this time is calculated by taking the average LD_{50} value for the lot from the third instar (L3) diet plug experiment and dividing it by 2.2 which is the average ratio of the comparison (from Table 7) of the mean lethal dose (L3 plug) and mean lethal concentration (L2 surface). This calculated LC_{50} "current" value is then compared to the original LC_{50} value as was done in Table 8 with the experimental values. This comparison gives the same ratio of 0.7 for lot 3 and lot 7 and a ratio of 0.8 (different from 0.4 the experimental value) for lot 9. This indicates that the third instar LD_{50} values can be used to generate a calculated LC_{50} value for second instar which agrees with the experimental value.

We give an example for this calculation. For Lot 3 the average LD_{50} value for third instars in our diet plug bioassays was 60 (Table 9). When 60 is divided by 2.2 (average ratio comparison from Table 7) the resulting value is 27. When the original LC_{50} value of lot 3, 17.9 (Table 8), is divided by 27 the value obtained is 0.7.

Table 9. Comparison of adjusted ratio of “original” median LC_{50} of TM BioControl-1® production lot samples with second instar Douglas-fir tussock moth larvae (GL-1 strain) with “current” median LD_{50} of the same virus lots[a]

Lot number	Average LD_{50} of L3 plug PIBs/µl	Average ratio adjustment[b] ÷ 2.2	Ratio of original LC_{50}/current LD_{50}
3	60	27	0.7
7	47.6	21.6	0.7
9	60.5	27.5	0.8

[a] LC_{50} values were determined for second instar larvae of the Goose Lake colony with the diet surface contamination. LD_{50} values were determined for third instar larvae of the same strain using the diet plug method.

[b] Average ratio adjustment factor of 2.2 is the average L3 plug/L2 surface bioassay comparison ratio among all virus samples tested (see Table 7).

Discussion

The original activity standardization bioassays, done on advance samples supplied by the processing companies, were done in Corvallis, OR, on second instar larvae using the diet surface contamination technique. In our bioassays, we used the diet plug inoculation technique on third instar larvae. The diet plug method is more accurate than the diet surface contamination bioassay, and it is much easier to work with older, third instar than second instar larvae. Data from these comparison experiments may provide a useful shortcut for forest managers in calculating acre-dose values if they are unable to do the preferred two-step acre-dose determination because of time or other limitations.

In our experiments, we used both second instar and third instar larvae from the same generation of the Goose Lake strain (the same strain was also used in the bioassays at the time of virus production, prior to storage), and administered the virus by: a) the diet plug method of inoculation; and b) the diet surface contamination method using the same samples of the same lots of stored TM BioControl-1® product.

The calculated LD_{50} values of the third instars in the diet plug bioassays were 3.6 to 4.9 times higher than LD_{50} values of the second instars in the diet plug bioassays (Table 5). This means it takes an average of 4.2 times more virus to kill 50% of the third instars than the second instar larvae. This is expected because, generally, older larvae require more virus to kill them. In addition, some degradation of the efficacy of the virus occurred in storage. This loss of efficacy also contributed to the higher LD_{50} for the third instar in our current bioassay.

Some of the LC_{50} data from the diet surface contamination bioassays had large 95% fiducial limits (at the $p<0.05$ level) which resulted in some comparisons which were not significantly different. Diet surface contamination bioassays are not as accurate as the diet plug bioassays because there is variation in the distribution of the virus on the diet surface, larvae have different feeding rates, and it is not known how much virus each larva had consumed. All these factors contribute to variation in the diet surface contamination bioassay data.

The LC_{50} values of the second instar diet surface experiment were 1.4 to 2.9 times higher than the LD_{50} values of the second instar in the diet plug experiment (Table 6). This indicates that it takes an average of 2.0 times more virus to kill 50% of the larvae with the diet surface contamination method than with the diet plug method.

The LD_{50} values in the third instar diet plug bioassay were 1.2 to 3.0 times higher than LC_{50} values of the second instar diet surface experiment (Table 7). This suggests that it would take an average of 2.2 times more virus to kill 50% of the third instar larvae in the diet plug method using the same lot of stored product compared to second instars in the diet surface contamination method.

Comparing the LC_{50} value from the original diet surface contamination bioassay, done at the time when the TM BioControl-1® was produced before packaging it for storage, with the results from the diet surface contamination bioassays we did, we found that the LC_{50} values now are higher after 7 and 14 years of storage of the samples, which suggests a decrease in potency of the product. We made this comparison by calculating the ratio of original LC_{50} over our current LC_{50} values. In both cases, second instar larvae of the Goose Lake strain of Douglas-fir tussock moth were used. Original LC_{50} values were calculated from data collected at day 12 post-inoculation. Current LC_{50} values were recalculated from data 12 days after inoculation for this comparison. These ratios are less than 1 (Table 8). The ratio of LC_{50} values for lot 3 and lot 7a was 0.7 while for lot 9c the ratio was 0.4. However, the LC_{50} value for sample #41 from lot 9 was higher and with disproportionately higher 95% fiducial limits than the other LC_{50} values for fresh, sample #7 lot 3 and #27 lot 7a (Fig. 1). This gave a lower ratio when compared to the original LC_{50} value, and probably is unreliable. As pointed out earlier, it is difficult to control the amount of virus consumed and differences in feeding behavior of the larvae that can occur with the diet surface contamination bioassays. We suggest that these factors account for some of the differences noted in the dosage ratio comparing the original bioassay results with the current for lot 9. Lot 3 was in storage for 14 years, Lot 7 for 10 years, and lot 9 for 7 years. This indicates a decrease in the potency of the virus product with storage time, but the decrease does not appear to be directly related to storage time (i.e. the relationship does not appear to be linear).

The 0.7 ratio of lot 3 and lot 7a suggests that the potency is 70% of what it was when the TM BioControl-1® was first produced. It should be noted that the difference may be due to the time in storage, but could also have been caused in part by changes in the Goose Lake strain colony over the years, or a combination of these. If the Goose Lake strain larvae became more resistant over time to TM BioControl-1®, then the LC_{50} values would be higher and the ratio compared to the original LC_{50} data would be less than 1. Martignoni and Iwai (1978) demonstrated the long-term (13 year) stability of the response of the GL-1 strain to virus bioassay, but since that time the GL-1 strain has been maintained for an additional 23 years of continuous laboratory culture without infusion of new genes from field populations. It is not known what effect, if any, two additional decades of inbreeding may have had on this insect colony. It is conceivable that maintaining the GL-1 strain for these additional years favors the survival of those larvae that were more "resistant" to OpNPV.

Conclusions

We found that for bioassays with the same TM BioControl-1® samples:

1) The calculated LD_{50} values of the third instars in the diet plug bioassays were 3.6 to 4.9 times higher than LD_{50} values of the second instars in the diet plug bioassays. This means it takes an average of 4.2 times more virus to kill 50% of the third instars than the second instar larvae.
2) The LC_{50} values of the second instar diet surface experiment were 1.4 to 2.9 times higher than the LD_{50} values of the second instar diet plug experiment. This indicates that it takes an average of 2.0 times more virus to kill 50% of the larvae with the diet surface contamination method. Thus, acre-dose values, because they were calculated from diet surface contamination, were unintentionally overestimated. This could, in part, account for the relatively high registered dose of TM BioControl-1® (2.5 x 10^{11} PIBs/ha) and may explain why reducing the registered dose in field-testing is logical.
3) The LD_{50} values in the third instar diet plug bioassay were 1.2 to 3.0 times higher than LC_{50} values of the second instar diet surface experiment. This suggests that it would take

an average of 2.2 times more virus to kill 50% of the third instar larvae using the diet plug method compared to second instars in the diet surface contamination method.

4) Current LC_{50} values for second instar larvae in the diet surface contamination bioassays are higher than original LC_{50} values, indicating that some decrease in potency of TM BioControl-1® occurred with time. However, this decrease in potency does not appear to be directly related to length of time.

Recommendations

Larval development in the field is not uniform. Although the aim of a control operation is to treat as early as possible, as first instar larvae disperse and start active feeding on the new growth, it may not be always be feasible to treat first instar larvae. Both second and third instar larvae can be successfully treated in suppression projects provided that the higher dose requirement of third instar larvae is taken into consideration. Ideally, the acre-dose determination should be done with a two-step bioassay. In the first or range-finding bioassay the activity of the stored product is tested and then the amount of product to be used per acre is determined in the second bioassay using larvae emerging from field-collected egg masses from the location(s) that will be treated.

We have compared LC_{50} values determined for second instar larvae using the diet surface inoculation method and LD_{50} obtained for third instar larvae with the more accurate diet plug method. The conversion factors we provide may be used to derive acre-dose determination if the second bioassay cannot be done with larvae emerging from field collected egg masses.

References Cited

Hadfield, J. S., and R. W. Magelssen. 1995. TM BioControl-1® production history and inventory status. USDA Forest Service Region 6 Bulletin, Corvallis, OR. 20 p.

Kalmakoff, J. 1980. Viruses, pp. 21-57. *In* J. Kalmakoff and J. Longworth (eds), Microbial Control of Insect Pests. Bulletin 228, New Zealand Dept. of Scientific and Industrial Research, Wellington, NZ.

Kaupp, W. J., and P. M. Ebling. 1990. Response of third, fifth, and sixth instar spruce budworm, Choristoneura fumiferana (Clem.), larvae to nuclear polyhedrosis virus. Can. Entomol. 122: 1037-1038.

Martignoni, M. E. 1978. Production, activity, and safety, pp. 140-147. *In* M. H. Brookes, R. W. Stark, and R. W. Campbell (eds.), The Douglas-fir tussock moth: a synthesis. USDA Technical Bulletin 1585, USDA Forest Service, Science and Education Agency, Washington, DC.

Martignoni, M. E. 1999. History of TM BioControl-1: the first registered virus-based product for control of a forest insect. Am. Entomol. 45: 30-37.

Martignoni, M. E., and P. J. Iwai. 1977. Peroral bioassay of technical-grade preparations of the Douglas-fir tussock moth nucleopolyhedrosis virus (Baculovirus). USDA Forest Service Research Paper PNW-222, Pacific Northwest Forest and Range Experiment Station, Portland, OR. 12 p.

Martignoni, M. E., and P.J. Iwai. 1978. Activity standardization of technical preparations of Douglas-fir tussock moth *Baculovirus*. J. Econ. Entomol. 71: 473-476.

SAS Institute. 1989-1996. SAS users guide: statistics, version 6.12. SAS Institute, Carey, NC.

Scott, D. W. 2000. Activity standardization and determination of acre dose of TM BioControl-1 preparation. Internal USDA Forest Service document.

Thompson, C. G., and L. J. Peterson. 1978. Rearing the Douglas-fir tussock moth. USDA Agriculture Handbook No. 520, Combined Forestry Pest Research and Development Program, Washington, DC. 20p.

Section 4

Evaluation of TM BioControl-1® Infection of Douglas-fir Tussock Moth Larvae Reared on Douglas-fir Foliage After Inoculation

B. Kukan[1] and I.S. Otvos[1]

[1] Natural Resources Canada, Canadian Forest Service, Pacific Forestry Centre, Victoria, BC, V8Z 1M5 Canada.

Introduction

As part of the collaborative research project, it was agreed that one set of bioassays would be conducted to test the efficacy of the TM BioControl-1® under more field-like conditions. Various chemicals in the host leaves and needles have been reported to affect the susceptibility of the insects to nucleopolyhedrovirus (NPV) infection (Felton et al. 1987, Keating et al. 1988, Keating et al. 1989, Felton and Duffey 1990, Hunter and Schultz 1993). We investigated if feeding on Douglas-fir (*Pseudotsuga menziesii* [Mirbel] Franco) foliage after inoculation affects the susceptibility of Douglas-fir tussock moth (*Orgyia pseudotsugata* [McDunnough]) larvae to TM BioControl-1® infection because no information was available on this topic.

Materials and Methods

Bioassay Protocol

Douglas-fir tussock moth larvae of the Goose Lake colony were used in this bioassay. Egg masses were gently teased apart and the eggs were decontaminated to reduce potential virus contamination, by washing them three times in 1% sodium hypochlorite (2% bleach) solution and rinsing them in distilled water after each wash. The decontaminated eggs were placed in sterile petri dishes and larvae were reared at 25°C±1°C, 50-60% RH with a photoperiod of 16:8 (L:D). Larvae were reared in groups of 10 on artificial diet (Thompson and

Peterson 1978). Newly molted third instar larvae (<24 h old) were selected for the experiment and starved for 16-20 h before the bioassay.

Virus Preparation

TM BioControl-1® samples used in this experiment were samples #7 from lot 3 stored for 14 years, #27 from lot 7 stored for 10 years and #41 from lot 9 stored for 7 years. These were three of the six samples used in the comparative study of the response of second and third instars to infection using the diet surface contamination and diet plug bioassays (Section 3). Twenty mg of these three samples was weighed and mixed with 20 ml of distilled water. Samples were stirred on a magnetic stirrer for 2 h and viral samples prepared in triplicate. One week prior to inoculation, serial dilutions were made and stored at 4°C until infection. Concentrations were chosen to determine, as accurately as possible, the median lethal dose (LD_{50}) and generally ranged from 16-240 polyhedral inclusion bodies (PIBs) per larva. Ideally, concentrations are selected to give approximately the LD_{30}, LD_{35}, LD_{45}, LD_{70} and LD_{75}, the recommended lethal dose values to aim for in an accurate LD_{50} determination (Robertson and Preisler 1992).

A fresh OpNPV virus sample, to be used as a positive control, was prepared prior to the bioassay by homogenizing 100 Goose Lake strain Douglas-fir tussock moth larvae, previously inoculated with OpNPV from lot 1 and reared until death, in 5 ml of distilled water. The fresh homogenate was filtered through cheesecloth, centrifuged once at 10 x g for 5 min and twice at 7100 x g for 20 min on an IEC centrifuge and resuspended in distilled water. PIB counts were obtained using a haemocytometer to quantify the virus. The fresh OpNPV sample was produced 1-2 weeks prior to use, and then serial dilutions prepared and stored at 4°C until the experiment. None of the serial viral dilutions were kept longer than 3 months.

Foliage Preparation

Two months prior to the experiment, potted Douglas-fir trees approximately 40-50 cm in height were placed in January in a greenhouse set at 20^0C±1°C to force flushing of the new growth. The new growth was sufficiently long in March for use in the experiment. The new growth, clipped off the tips of the branches into 2.5 cm lengths, was used to feed the Douglas-fir tussock moth larvae. The new growth was decontaminated in a 10% bleach solution for 5 minutes and then rinsed twice with water. The foliage was placed in a plastic bag and stored at 4^0C until use after inoculation.

Inoculation

The diet plug inoculation bioassay technique we used was similar to that of Kaupp and Ebling (1990). The diet plug bioassay is superior to diet surface contamination because it eliminates any variation in the distribution of PIBs on the diet surface and eliminates any differential feeding rates among the larvae. Only those larvae that eat the whole diet plug were used in the experiment. One μl of each viral dilution or control (distilled water) was added to a small diet plug (3-4 mg) inside each well of a 24-well tissue culture plate. Previous experiments have shown that this size diet plugs were large enough to fully absorb the 1 μl of liquid and most larvae ate this size diet plug in 24 h. Five virus concentrations were tested for each of the three TM BioControl-1® samples and the fresh OpNPV sample (positive control). For each viral dilution and control 48 larvae were tested. In each bioassay replicate there were five viral dilutions for each of the four virus samples (three stored and one fresh), and one control, requiring 1,008 larvae per replicate. There were three replicates, thus a total of 3,024 larvae were used in the experiment ([{48 larvae x 5 concentrations x 4 virus} + {48 larvae x 1 control}] x 3 replicates).

The replicates were done on three successive days. We used 1-day old third instar Douglas-fir tussock moth larvae, from the Goose Lake laboratory colony. Larvae were starved for 24 h prior to inoculation. Immediately after inoculation of the diet plugs, one third instar larva was placed into each well to feed on the treated diet plug. Larvae were held in darkness for 24 h with the inoculated diet plug at 25 °C ±1°C, 50-60% RH. Only larvae that consumed the entire diet plug were used in the bioassay and were placed in individual cups (Solo P100, Solo Cup Co. Urbana, IL 61801-2895) with fresh, untreated Douglas-fir foliage (new shoots) and returned to the same growth chamber. Larvae that did not consume the entire diet plug were discarded. Because of the high virulence of OpNPV, the larvae were reared individually after inoculation to avoid cross-infection. In addition, separate growth chambers, set at the same rearing conditions, were maintained for the control and viral infected insects to guard against viral transmission to the control insects. Foliage (forced new growth) was changed at least twice weekly or more often if it appeared to dry out. Larvae were reared for 21 days post-inoculation and were examined and the mortality recorded daily. Only larvae that died from OpNPV infection, as determined by gross pathology or microscopic examination, were included in the analysis.

Data Analysis

Using the larval mortality data, dosage mortality curves and LD_{50} values in PIBs with associated 95% fiducial limits were calculated using PROC PROBIT analysis (SAS Institute 1989-1996). Each replicate at day 14 post-inoculation (and day 21) was tested as a separate preparation to verify that there was no difference in the replicates. No differences were found so data for the three replicates were combined. Estimates of LD_{50} for each TM BioControl-1® sample and the fresh OpNPV sample were calculated. LD_{50} values at day 14 and day 21 were compared to see if lethal doses changed over time. LD_{50} values were examined for significant differences (no overlap of the 95% fiducial limits).

Variation of LD_{50} among TM BioControl-1® samples was examined. There were no significant differences ($p<0.05$) between LD_{50} values at day 14 compared to day 21. Therefore, we used day 14 data in the analysis.

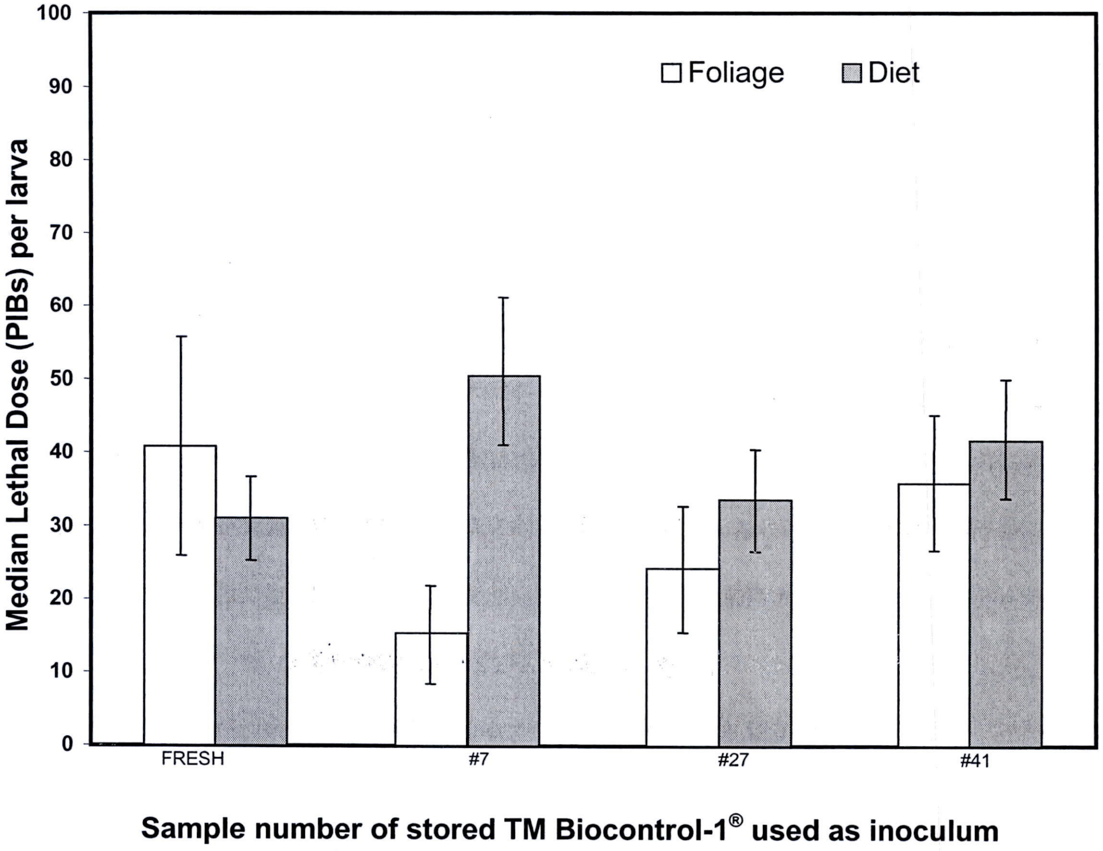
Experiments were designed to calculate the median lethal dose (LD_{50}). Concentrations were selected to give approximately 30%, 35%, 45%, 70% and 75% mortality, as recommended by Robertson and Preisler (1992). For each bioassay, the concentrations chosen were based on previous experiments as a best guess of what was appropriate. Results from this foliage bioassay were compared with those from another bioassay conducted concurrently that examined the laboratory response of second instar and third instar Douglas-fir tussock moth to OpNPV (reported in Section 3) using the same three virus samples (#7, #27 and #41). In that bioassay, an additional 3,024 third instar larvae, divided the same way as for the foliage bioassay among the treatments and controls, had been reared both before and after inoculation on artificial diet only. These larvae were inoculated the same way, and treated the same way following inoculation, as the foliage reared insects, except that they were reared on diet instead of foliage. Diet was changed at least weekly, or more often if needed. We used the results of rearing the additional 3,024 third instar larvae on diet for comparison with the 3,024 larvae reared on foliage after inoculation. Thus, for the results reported in the current study one-half of the larvae after inoculation were reared on decontaminated "forced" new growth of Douglas-fir while the other half of the larvae were reared on artificial diet after inoculation.

Median lethal dose values for the fresh OpNPV and samples of the stored TM BioControl-1® lots (#7, #27 and #41) obtained from the foliage bioassay were compared to values obtained for the same TM BioControl-1® samples in the other concurrent study using diet described above.

Results

There was no difference in the LD_{50} values of the fresh samples between the larvae reared on foliage after infection and those reared on diet in this study (Fig. 1). In addition, there was no significant difference in the LD_{50} values of two of the TM BioControl-1® samples (#27 and #41, stored for 10 and 7 years, respectively) among larvae reared on foliage after infection and those reared on diet. However, there was a significant difference (foliage versus diet) in the LD_{50} values of the third TM BioControl-1® sample, #7. This sample came from lot 3 that was stored the longest (14 years). The larvae reared on foliage after infection had a lower LD_{50} value than the larvae reared on artificial diet. This suggests that the larvae were more susceptible to virus infection when their diet had been switched to foliage after inoculation, i.e. required less PIBs to kill them.

Fig. 1. Comparison of the median lethal dose (LD_{50}) values in PIBs per larva of the Douglas-fir tussock moth larvae (Goose Lake 1 strain) fed Douglas-fir foliage and artificial diet, respectively, after infection using the diet plug method with three samples of stored TM Biocontrol-1® and a fresh sample of OpNPV.[a, b]



[a] The bars indicate 95% fiducial limits.

[b] Samples #7 (lot 3), #27 (lot 7) and #41 (lot 9) have been stored for 14, 10 and 7 years, respectively.

Discussion

Changes in the food of some immature Lepidoptera have been reported to alter their susceptibility to nucleopolyhedrovirus infection (Felton et al. 1987, Keating et al. 1988, 1989; Felton and Duffey 1990). Data from these studies indicate that a change in food source or age of foliage can affect the susceptibility of the insect to viral infection. Felton et al. (1987) found that rutin and chlorogenic acid in the tomato plant, *Lycopersicon esculentum* Mill., were identified as potential sources of host plant resistance against the tomato fruitworm, *Heliothis zea* (*Helicoverpa zea* [Boddie])[a], and significantly inhibited the infectivity of the tomato fruitworm nucleopolyhedrovirus HzSNPV. Ingestion of these two phytochemicals significantly prolonged survival time of virus infected larvae resulting in higher LD_{50} values. In a later study, Felton and Duffey (1990) reported that chlorogenic acid in the presence of polyphenol oxidase is rapidly oxidized to chlorogenoquinone when foliage is damaged during feeding by *H. zea*. They concluded that the chlorogenoquinone covalently bound to occlusion bodies of HzSNPV and significantly reduced their digestibility and solubility under alkaline conditions making the larvae less susceptible to infection.

The susceptibility of gypsy moth, *Lymantria dispar* (L.), to LdNPV has been shown to be affected by change in host plants (Keating and Yendol 1987, Keating et al. 1988). These two studies have shown that gypsy moth larvae consuming the virus on oak (*Quercus* spp.) foliage had lower mortality than did larvae consuming the virus-contaminated foliage of aspen (*Populus* spp.). Larvae on oak were less susceptible to infection while those on aspen were more susceptible. In a later study, Keating et al. (1990) concluded that diet pH may affect virus activity by changing midgut pH and altering the rate and location of virion release in the midgut lumen. The ability of gypsy moth virus and other baculoviruses to cause fatal infection was reported to be reduced significantly when larvae consume foliage high in polyphenols (Keating et al. 1988,1989; Hunter and Schultz 1993). The increasing levels of tannin in oak leaves as the leaves age have been reported to negatively affect infection by LdNPV in the gypsy moth (Barbosa and Krischik 1987, Schultz 1989, Keating et al. 1989, Hunter and Schultz 1993). Similarly, Richter et al. (1987) found differences in susceptibility of the fall armyworm, *Spodoptera frugiperda* (J.E. Smith), to a nucleopolyhedrovirus (SfNPV) when larvae were fed different host plants (corn, signalgrass, brown-top millet, soybeans, bermudagrass, ryegrass or sorghum). They concluded that the differences were related to the fall armyworm being stressed by consumption of less suitable host plants (sorghum, bermudagrass, soybeans) which resulted in greater susceptibility to viral infection. The virulence and infectivity of nucleopolyhedrovirus was also shown to be affected by the dietary habits of *Helicoverpa armigera* Hubner (Lepidoptera: Noctuidae) (Ramarethinam et al. 1998).

---

[a] The name *Helicoverpa zea* (Boddie) is the currently accepted name of the tomato fruitworm listed in Bosik (1997).

In the study reported here, we infected third instar Douglas-fir tussock moth with fresh OpNPV and stored TM BioControl-1® samples on diet plug, and then reared one-half of the infected larvae on Douglas-fir foliage and the other half on artificial diet. Our results indicated that in all but one case there was no significant difference in the LD_{50} values of larvae given Douglas-fir foliage and those given artificial diet after infection with either fresh virus or samples of stored TM BioControl-1®. The only significant difference occurred with the sample stored for 14 yr (#7). However, the LD_{50} was also lower, though not significantly, when larvae were reared on foliage after infection with the samples of the other two stored samples (#27, #41). Larvae were equally susceptible to the fresh virus when given either diet or Douglas-fir foliage after infection. One of the TM BioControl-1® samples did show a significantly lower LD_{50} with larvae given Douglas-fir foliage. This may indicate greater susceptibility to the virus with the foliage. However, it seems more likely that the larvae did not adjust well to the switch from diet to foliage after infection, which may have stressed the larvae thus making them more 'susceptible' to infection. The Douglas-fir trees used in the experiment were reared in pots and placed in greenhouse conditions for 2 months for this study. It is not known what effect, if any, this may have had on the phytochemicals in the plants. Even under natural conditions, Douglas-fir may not produce phytochemicals that would affect the interaction between OpNPV and its host, the Douglas-fir tussock moth. Therefore, the susceptibility of the Douglas-fir tussock moth larvae to OpNPV would not be altered/changed. This is important because our feeding data showed (without analyzing the chemical composition of the host foliage) that in the case of Douglas-fir tussock moth, whose primary host is Douglas-fir, the foliage of the host does not appear to have chemical compounds that would interfere with the infection process of OpNPV after inoculation. This means that laboratory bioassays in which the inoculated insects are reared on artificial diet are representative of what is likely to happen in the field where the insects feed on Douglas-fir (i.e. the effectiveness of the virus is not reduced by the chemicals in the foliage).

In conclusion, experiments done in the laboratory using insects reared on artificial diet gave a good indication of the efficacy of the virus on Douglas-fir tussock moth larvae. It is not necessary to incur the extra cost and time to feed Douglas-fir foliage to the inoculated larvae (after inoculation) to indicate efficacy in the field, until such time that the chemical analysis of the foliage would indicate sufficient chemical differences during shoot elongation when a suppression project would normally be conducted against the Douglas-fir tussock moth.

References Cited

Barbosa, P., and V. A. Krischik. 1987. Influence of alkaloids on feeding preference of eastern deciduous forest trees by the gypsy moth, *Lymantria dispar*. Am. Nat. 130: 53-69.

Bosik, J. J. 1997. Common names of insects and related organisms. Entomological Society of America, Lanham, MD.

Felton, G. W., and S. S. Duffey. 1990. Inactivation of baculovirus by quinines formed in insect-damaged plant tissues. J. Chem. Ecol. 16: 1221-1236.

Felton, G. W., S. S. Duffey, P. V. Vail, H. K. Kaya, and J. Manning. 1987. Interaction of nuclear polyhedrosis virus with catechols: potential incompatibility for host-plant resistance against Noctuid larvae. J. Chem. Ecol. 13(4): 947-958.

Hunter, M. D., and J. C. Schultz. 1993. Induced plant defenses breached? Phytochemical induction protects an herbivore from disease. Oecologia 94: 195-203.

Kaupp, W. J., and P. M. Ebling. 1990. Response of third, fifth, and sixth instar spruce budworm, *Choristoneura fumiferana* (Clem.), larvae to nuclear polyhedrosis virus. Can. Entomol. 122: 1037-1038.

Keating, S. T., and W. G. Yendol. 1987. Influence of selected host plants on gypsy moth (Lepidoptera: Lymantriidae) larval mortality caused by a baculovirus. Environ. Entomol. 16: 459-462.

Keating, S. T., W. G. Yendol, and J. C. Schultz. 1988. Relationship between susceptibility of gypsy moth larvae (Lepidoptera: Lymantriidae) to a baculovirus and host plant foliage constituents. Environ. Entomol. 17(6): 952-958.

Keating, S. T., W. J. McCarthy, and W. G. Yendol. 1989. Gypsy moth (*Lymantria dispar*) larval susceptibility to a baculovirus affected by selected nutrients, hydrogen ions (pH), and plant allelochemicals in artificial diets. J. Invertebr. Pathol. 54: 165-174.

Keating, S. T., J. C. Schultz, and W. G. Yendol. 1990. The effect of diet on gypsy moth (*Lymantria dispar*) larvae midgut pH, and its relationship with larval susceptibility to a baculovirus. J. Invertebr. Pathol. 56: 317-326.

Ramarethinam, S., S. Marimuthu, B. Rajagopal, and N. V. Murugesan. 1998. Relative changes in the virulence and infectivity of the nuclear polyhedrosis virus in relation to the dietary habit of *Helicoverpa armigera* Hubner (Lepidoptera: Noctuidae). J. Appl. Entomol. 122: 623-628.

Richter, A. R., J. R. Fuxa, and M. Abdel-Fattah. 1987. Effect of host plant on the susceptibility of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to a nuclear polyhedrosis virus. Environ. Entomol. 16: 1004-1006.

Robertson, J. L., and H. K. Preisler. 1992. Pesticide Bioassays with Arthropods. CRC Press, Boca Raton, FL.

SAS Institute. 1989-1996. SAS users guide: statistics, version 6.12. SAS Institute, Carey, NC.

Schultz, J. C. 1989. Tannin-insect interactions, pp. 417-443. *In* R. W. Hemingway and J. Karchesy (eds.), The Chemistry and Significance of Condensed Tannins. Plenum Press, New York, NY.

Thompson, C. G., and L. J. Peterson. 1978. Rearing the Douglas-fir tussock moth. USDA Agriculture Handbook No. 520, Combined Forestry Pest Research and Development Program, Washington, DC. 20p.

Section 5

Effects of long-term storage on the stability of OpNPV DNA contained in TM BioControl-1®

C. Reed and I. S. Otvos

Natural Resources Canada, Pacific Forestry Centre, Victoria, BC, V8Z 1M5, Canada

Abstract

Orgyia pseudotsugata nucleopolyhedrovirus (OpNPV) DNA was extracted from samples representing 10 lots of TM BioControl-1® stored for 5 to 15 years and digested with *Bgl* II, *Pst* I and *Sal* I restriction enzymes. No restriction fragment length polymorphisms were observed in any of the samples, and there was no evidence of DNA degradation. These results suggest that the long-term cold storage (-10°C) of TM BioControl-1® had no adverse effect on the quality of the OpNPV genomic DNA contained in TM BioControl-1®.

In samples from lot numbers 4a, 5b and 6, ten segments of nucleic acid ranging in size from 0.6 kb to 4.4 kb were observed in addition to the expected >23 kb OpNPV band. The electrophoretic profile of these segments was characteristic of *Orgyia pseudotsugata* cytoplasmic polyhedrosis virus (OpCPV), and RNase A/DNase I treatment showed that the nucleic acid contaminants were composed of RNA. This indicates that lots 4a, 5b and 6 of TM BioControl-1® contain OpCPV as well as OpNPV.

Introduction

TM BioControl-1® is a biological insecticide that was produced and registered by the United States Department of Agriculture (USDA) Forest Service for the purpose of suppressing outbreaks of *Orgyia pseudotsugata* (McDunnough) (Douglas-fir tussock moth). TM BioControl-1® contains the active ingredient OpNPV, a nucleopolyhedrovirus effective in controlling Douglas-fir tussock moth outbreaks because it is highly pathogenic to the Douglas-fir tussock moth (Martignoni 1999).

Douglas-fir tussock moth virus was propagated in late instar Douglas-fir tussock moth larvae from a laboratory colony, called the Goose Lake colony, at the USDA Forest Service Pacific Northwest Research Station, Forestry Sciences Laboratory, Corvallis, Oregon from 1980 to 1992. The virus-killed larvae were frozen and sent to companies for processing to recover virus from the dead larvae and thus produce TM BioControl-1®. This processing was conducted from 1985 until the last lot processed was placed in vacuum-packed packages for storage in late January 1995 (Hadfield and Magelssen 1995). The exclusive source of virus for all of the production lots of TM BioControl-1® was a strain of the multinucleocapsid OpNPV (OpMNPV) called MEM-75-STANDARD, which was established by the USDA Forest Service in 1978. The propagation and amplification of OpMNPV followed the "seed-lot system" used by Fox et al. (1943) for the production of a strain of yellow fever virus. This system was implemented to avoid contamination of the pathogen, to ensure consistency between lots, and to minimize the chance of the virus mutating. No more than two passages of virus separated the produced lots from the primary "seed lot" of the MEM-75-STANDARD strain. The virus was propagated in a laboratory strain of Douglas-fir tussock moth free of endogenous viral infections. This strain, the GL-1 strain of *O. pseudotsugata*, is an inbred colony of insects established from eggs collected near Goose Lake in northern California and maintained at the Corvallis Forestry Sciences Laboratory until 1995 (Martignoni 1999). After 1996 the GL-1 strained was maintained at the Pacific Forestry Centre, Canadian Forest Service, Natural Resources Canada, Victoria, BC.

Production of TM BioControl-1® from infected *O. pseudotsugata* larvae required a method that concentrated the viral inclusion bodies and excluded most of the unwanted insect debris. Larval cadavers were homogenized in water and the slurry was centrifuged to concentrate the polyhedral inclusion bodies (PIBs). The virus-rich sediments were lyophilized, screened to a fine powder, and vacuum-packaged for long-term storage (Martignoni 1999). Although the USDA Forest Service could rear large numbers of infected larvae, they did not possess the equipment necessary to process large quantities of the final TM BioControl-1® product. In 1983, the USDA- Agricultural Research Service processed the first batch of TM BioControl-1® from 18 kg of infected larvae. A portion of this batch was used for research, and the remainder, 1680 acre-doses (representing lot 1), was returned to the USDA Forest Service. Of that lot only 480 acre-doses remained, which have been in cold storage since 1985 (Hadfield and Magelssen 1995), at the start of this cooperative work in 1998.

Large scale processing of TM BioControl-1® was subsequently contracted out to three different companies (Table 1). The first contract was awarded to Reuter Laboratories Inc. in 1986, and this company processed 147,600 acre-doses, comprising lot numbers 2, 3, 4 and 5. The USDA Forest Service awarded a processing contract to Espro Inc. in 1988, and this company processed 170,566 acre-doses comprising lot numbers 6 and 7. In 1990 Espro Inc. was acquired by Crop Genetics Inc., and this company continued the processing contract for the USDA Forest Service. Crop Genetics produced 108,784 acre-doses comprising lot numbers 8, 9 and 10. Virus production ceased in 1992 after an adequate supply of TM BioControl-1® had been produced and stored (Hadfield and Magelssen 1995).

Table 1. Amounts and storage dates of TM BioControl-1® processed from 1985 to 1992[a]

Lot #	Processing company	Amount (acre-doses)	Date stored
1	Agri. Res. Service	480	1985
2	Reuter Labs Inc.	18,000	1986
3	Reuter Labs Inc.	13,000	1986
4a	Reuter Labs Inc.	24,800	1986
4b	Reuter Labs Inc.	33,000	1986
5a	Reuter Labs Inc.	10,800	1986
5b	Reuter Labs Inc.	26,000	1986
6	Espro Inc.	17,383	1989
7a	Espro Inc.	48,300	1990
7b	Espro Inc	50,000	1990
7c	Espro Inc	50,000	1990
8a	Crop Genetics Inc.	690	1991
8b	Crop Genetics Inc.	68,000	1991
9b	Crop Genetics Inc.	5,784	1993
9c	Crop Genetics Inc.	9000	1993
10a	Crop Genetics Inc.	1,000	1995
10b	Crop Genetics Inc.	24,000	1995

[a] Information taken from Hadfield and Magelssen 1995

The purpose of this specific investigation was to examine the stability or possible degradation and genetic variation of the OpNPV genomic structure contained within the stored TM BioControl-1® samples.

Materials and Methods

Extraction of OpNPV and OpCPV from TM BioControl-1®

Seventeen samples of TM BioControl-1® were obtained from the USDA Forest Service as a source of OpNPV DNA. These samples were taken from all of the package sizes (one to three) produced for each of the 10 lots of TM BioControl-1® processed from 1985 through 1995 (Table 1), with the exception of two packages (lots 1 and 8a) that each contained less than 700 total acre-doses. The entire range of time that TM BioControl-1® was stored (5 to 15 years) was covered by these 17 samples.

To extract OpNPV DNA, 0.5-1.0 g of each TM BioControl-1® sample was suspended in 20 ml of sterile distilled water. The suspension was centrifuged at 2000*g* for 10 min. The supernatant was removed and the pellet resuspended in 20 ml of sterile distilled water, centrifuged at 1300*g* for 10 min, and the supernatant discarded. The upper white layer of the pellet was carefully resuspended in 1.5 ml of sterile distilled water and transferred to a microfuge tube. Samples were centrifuged at 14,000*g* for 15 min to pellet the polyhedral inclusion bodies (PIBs). The pellet was washed three times with sterile TE (10 mM Tris-HCl pH 7.6; 1 mM EDTA, pH 8.0). The final pellet was resuspended in 0.1 M sodium carbonate buffer (pH 10) and incubated at room temperature for 20 min to dissolve the polyhedra. Samples were centrifuged at 1000*g* for 5 min to pellet any large debris, and the supernatant, containing the virions, was transferred to a fresh microfuge tube and centrifuged at 18,000*g* for 30 min. The pellet was suspended in 500 µl of TES (100 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 8; 0.1% SDS), 200 µg/ml proteinase K was added, and the samples were incubated overnight at 55°C. Proteins were separated from viral DNA with two phenol: chloroform: isoamyl alcohol (25:24:1) extractions, and the DNA was further purified and concentrated using the GENECLEAN kit (Bio 101 Inc.). One µl of each sample was run on a 0.6% agarose gel containing 0.2% synergel (Diversified Biotech) stained with 100 ng/ml ethidium bromide. Genomic OpNPV DNA was isolated from each production lot and digested (cut) with a series of restriction enzymes. The resulting OpNPV genomic profiles were compared to profiles of MEM-75-STANDARD DNA and Douglas-fir tussock moth larval host DNA digested in the same manner. DNA concentrations were estimated by comparing the sample band intensities to those of 125 ng of Lambda DNA digested with *Hin*d III.

Extraction of OpNPV from Infected Douglas-fir Tussock Moth Larvae

MEM-75-STANDARD was provided to us by Dr. Iral Ragenovich, USDA Forest Service, Portland, OR (Region 6), for the comparison of the samples from the stored product. To obtain enough sample of MEM-75 to use as a positive control, newly molted third instar Douglas-fir tussock moth larvae (Goose Lake strain) were inoculated with a dilute sample of MEM-75-STANDARD (75-F-06) obtained from the USDA Forest Service. This single passage of the standard allowed us to minimize the amount used of the extremely valuable standard left over from the time of production, which may be required later for production of additional TM-BioControl-1® should the decision be made to restart its production. The concentration of PIBs in MEM-75-F-06 was calculated using a hemocytometer and 5 µl aliquots of the stock solution. The PIB count was determined to be 1.63×10^7 PIBs/ml. Ten µl (16,300 PIBs) of a 1:10 dilution of the MEM 75-F-06 stock was applied evenly to the top of each artificial diet cube (measuring 6 mm × 6 mm × 3 mm [L×W×H]) and allowed to soak in. The diet cube was placed in a 150 mm x 15 mm petri dish (Fisher Scientific) containing 10 fifth instar *O. pseudotsugata* larvae. When required, the cube was rehydrated with 10 µl of dH_20 to ensure that the larvae consumed as much of the virus contaminated diet cube as possible. Insects were reared until death, at which point the petri dishes were placed at –20°C and stored until required.

The stored dead larvae were ground to a fine powder in a liquid nitrogen-cooled mortar and pestle with 0.5 ml STE-C buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0; 50 mM NaCl; 10 mM cysteine). The powder was transferred to microfuge tubes containing 1.0 ml of STE-C with 0.1% SDS and 0.06 mg/ml DNase I, and the tubes were incubated with gentle shaking at room temperature for 20 min. The samples were then centrifuged at 150*g* for 3 min to pellet insect debris. The supernatant was transferred to a fresh microfuge tube and centrifuged at 18,000*g* for 15 min to pellet the PIBs. The pellet was washed three times with TE buffer (pH 7.6), and the final pellet was resuspended in 500 µl of 0.1 M sodium carbonate buffer (pH 11) and incubated with gentle shaking at room temperature for 20 min. The virions were pelleted by centrifugation at 18,000*g* for 30 min. The pellet was resuspended in 500 µl of TES followed by the addition of 200 µg/ml Proteinase K and incubated overnight at 55°C. Viral DNA was purified, concentrated, and its concentration estimated as described for the isolation of viral DNA from TM BioControl-1®.

Extraction of Host DNA

Disease-free *O. pseudotsugata* egg masses were obtained from the laboratory colony of Goose Lake strain (GL-1) and reared on virus-free artificial diet (Thompson and Peterson 1978) at the Pacific Forestry Centre since late 1994. Insects were reared until the third larval instar, at which point they were removed from the growth chamber and stored at –20°C. These third instar larvae were used for the extraction of non-infected *O. pseudotsugata* DNA.

Frozen larvae were ground to a fine powder with a liquid nitrogen-cooled mortar and pestle. One ml of lysis buffer (10 mM Tris-HCl, pH 8.5; 5.0 mM EDTA; 0.2% SDS; 200 mM NaCl; 200 µg/ml Proteinase K) was added to each powdered larva and the slurry incubated for several hours at 55°C. Following incubation, large debris were pelleted by a brief centrifugation. DNA was purified, concentrated, and its concentration estimated as described above.

Digestion of Viral Extracts and Southern Blots

OpNPV DNA extracted from the samples of TM BioControl-1®, total genomic *O. pseudotsugata* DNA, and MEM 75-F-06 viral DNA were compared using restriction analysis. Twenty to 50 ng of DNA was digested with each of the restriction enzymes *Bgl*

II, *Pst* I and *Sal* I (NEB) overnight at 37°C. Each digest was run in a 15cm x 20cm 0.6% agarose/1x TAE gel containing 0.2% synergel (diversified biotech) and 100 ng/ml ethidium bromide at 35 volts for 18 h. Digested DNA was passively transferred to nylon membranes (Hybond-N$^+$) according to manufacturer's protocol (Amersham Pharmacia Biotech).

Preparation of Digoxigenin-labeled NPV Probe

OpNPV genomic DNA was extracted from TM-BioControl-1® as described above. One hundred ng of DNA was heat-denatured by incubation at 94°C for 10 min and immediately cooled on ice. To synthesize the DIG-labeled probe, denatured DNA was mixed with 151.6 µg/ml random hexanucleotides, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, and 0.35 mM DIG-dUTP. Two units of labeling grade Klenow (DNA Polymerase I) was added and the reaction mixture was brought to a volume of 20 µl with sterile distilled water and incubated overnight at 37°C. Quantification of labeled probe was conducted according to the colourmetric detection protocol included in the DIG Labeling and Detection kit (Boehringer Mannheim, Cat. No 1093657).

Chemiluminescent Detection of Southern Blots

Prehybridization and hybridization reactions were done in 50 ml Falcon tubes, with one blot in each tube. Blots were prehybridized for 2 hours at 65°C in standard hybridization buffer (5x SSC; 0.1% N-lauroyl-sarcosine; 0.02% SDS; 1% blocking reagent; Boehringer Mannheim). Digoxigenin-labeled genomic OpNPV DNA was denatured for 10 min in a boiling water bath, immediately cooled in an ice bath, and 10 ng/ml was added to fresh hybridization solution. Prehybridization solution was removed from the tubes and 20 ml of hybridization solution was used for each 120 cm^2 blot. Blots were hybridized overnight at 65°C. Following hybridization, the solution containing the probe was transferred to a fresh 50 ml tube and stored at –20°C for use in subsequent hybridization reactions. The blots were washed twice at room temperature in 2x SSC, 0.1% SDS for 15 min each wash, then twice at room temperature in 0.1x SSC, 0.1% SDS for 30 min each wash. Detection of the digoxigenin-labeled DNA was conducted according to the manufacturer's protocol (Boehringer-Mannheim) and visualized with exposure to Bio-Max X-ray film (Eastman Kodak, Inc.).

RNase A digestion of CPV samples

To confirm that the contaminant nucleic acids detected in lots 4a, 5b, and 6 were RNA, the DNA extracts from these samples were subjected to RNase A (Sigma) or DNase I (Sigma) digestion. Replicates of CPV-containing OpNPV DNA extract were digested with 1 unit/ml of RNase A, 1 unit/ml DNA or distilled H_2O for 20 min at 37°C. Digests were run through a 0.6% agarose/0.2% synergel gel containing 100 ng/ml ethidium bromide. Results were visualized by UV illumination and photographed.

Results and Discussion

Physical Properties of TM BioControl-1®

The various lots of TM BioControl-1® differed quite markedly in their physical characteristics. While these differences do not likely affect the efficacy of the product, they should be noted nonetheless.

Lot 1, produced by the USDA Forest Service, was very coarse. It appeared to be a rough mixture of relatively large insect debris intermixed with a more homogeneous coarse powder. This product was so coarse that it was necessary to grind it with a mortar and pestle in order to facilitate adequate sedimentation of the PIBs for extraction of the viral DNA.

The lots produced by Reuter Labs. Inc., lot numbers 2 through 5, appeared fairly homogeneous in texture. Large insect debris was not readily apparent, and the powdered component was finer than lot 1. Samples from lot numbers 2 through 5 did not require additional grinding with a mortar and pestle in order to extract viral PIBs, although additional grinding did aid in the recovery of PIBs.

Lot numbers 6 through 10, processed by Espro/Crop Genetics Inc., were finer in texture than all of the other lots. These lots were very finely ground, and no large insect debris was apparent. Samples from these lots required no additional grinding with a mortar and pestle to aid in PIB recovery.

Extraction of viral DNA from the various samples of TM BioControl-1® yielded varying DNA concentrations, even though the same weight of sample was used for each extraction. Due to losses of DNA during the extraction and purification process, DNA recovery is not an effective way to quantify viral concentration in samples of TM BioControl-1®. However, in some cases, the differences in DNA recovered from lot to lot were larger than what could be reasonably be accounted for as experimental loss. This suggests that the quantity of PIBs (and virions) is not uniform from lot to lot.

Genomic Variation in TM BioControl-1®

Digestion of DNA with a restriction endonuclease (REN) produces a series of specific DNA fragments. Each REN produces a different series of fragments from the same DNA sample. Separation of the fragments by electrophoresis produces a characteristic restriction fragmentation pattern, or restriction fragment length polymorphism (RFLP) profile. This profile can be used to identify an individual or to compare individuals within a population. When DNA profiles from two or more individuals are different, they are said to contain polymorphisms. Polymorphisms are defined as a difference of at least one fragment in the RFLP profile of the sample DNA compared with that of the control DNA.

Seventeen samples of OpNPV DNA from 10 different production lots of TM BioControl-1® were extracted and each sample individually digested with *Bgl* II, *Pst* I and

Sal I restriction enzymes. The digested DNA was separated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with total genomic DNA. The DNA profiles from each lot were compared to identify any polymorphisms in the viral genomes. Digests of OpNPV DNA from all 17 samples with *Bgl* II, *Pst* I and *Sal* I did not show any polymorphisms when hybridized with total genomic OpNPV DNA. Fragment sizes produced from REN digestions were estimated by comparing the mobility of viral DNA fragments to the relative mobility of Lambda phage DNA cut with the restriction enzyme *Hin*d III.

Analysis of Bgl II RFLPs.

Digestion of TM BioControl-1® samples with *Bgl* II produced nine observable fragments ranging in size from 0.9 kb to greater than 25.5 kb (Fig. 1). No polymorphisms were detected; all of the samples shared the same RFLP profile as the MEM positive control. The RFLP profiles in the TM BioControl-1® samples and the RFLP profile of the MEM-75-STANDARD DNA were consistent with previous findings (Laitinen 1995, Miller and Dawes 1978) in both size and number of fragments observed.

Table 2. Estimated molecular weights, in kilobases, of OpNPV fragments generated by digestion with *Bgl* II restriction enzyme

Fragment	Estimated molecular weight	Reported molecular weight[a]
1	25.5	23.0+
2	24.3	23.0+
3	21.8	20.0
4	5.8	6.0
5	5.0	5.0
6	3.9	4.0
7	2.9	3.0
8	1.8	1.8
9	1.0	0.9

[a] Reported values for comparison taken from Laitinen (1995).

Analysis of Pst I RFLPs.

Twenty resolvable fragments, ranging from 0.9 kb to 6.7 kb, were identified in the TM BioControl-1® samples following *Pst* I digestion and hybridization with total genomic OpNPV DNA (Fig. 2). There were no observable polymorphisms in any of the TM BioControl-1® samples; all samples showed the same RFLP profile as that of the control DNA (MEM-75-STANDARD). The molecular size range and the separation profile of the control and the samples were consistent with results observed by Laitinen (1995) (Table 3).

Table 3. Estimated molecular weights, in kilobases, of OpNPV fragments generated by digestion with *Pst* I restriction enzyme

Fragment	Estimated molecular weight	Reported molecular weight[a]
1	6.4	6.5
2	5.4	5.5
3	4.8	5.0
4	4.3	4.5
5	4.1	4.1
6	3.6	3.8
7	3.2	3.5
8	2.7	2.8
9	2.4	2.4
10	2.2	2.1
11	2.1	1.9
12	2.0	1.8
13	1.8	1.6
14	1.5	1.4
15	1.3	1.3
16	1.1	1.2
17	1.0	1.1
18	0.8	0.9

[a] Reported values taken from Laitinen (1995).

Analysis of Sal I RFLPs.

Digestion of OpNPV DNA samples with *Sal* I produced essentially the same electrophoretic profiles for all 17 samples and the control MEM DNA (Fig. 3). For most of the samples and the control DNA, *Sal* I digestion produced 18 fragments, ranging in size from 0.75 Kb to 14 Kb (Table 4). The number and molecular weights of these fragments were consistent with previously reported findings for *Sal* I digestion of OpNPV DNA (Laitinen 1995, Miller and Dawes 1978). However, samples of TM BioControl-1® from lots 7a, 7b, and 7c resolved 20 fragments when digested with *Sal* I. The two additional fragments had molecular weights of 11.0 kb and 9.4 kb. The other 18 fragments correlated well with the 18 resolved in the other TM BioControl-1® samples and the control DNA. The two additional fragments were visible in the Southern blot exposure but not in the photograph of the gel taken before the transfer of the DNA onto a nylon membrane (not shown). Therefore, the concentration of these two fragments must have been very low. The fragments were probably visible in the Southern blot exposure but not in the photograph of the gel because the chemiluminescent detection system is more sensitive than photography. It is unlikely that the two fragments constitute a polymorphism, because a polymorphism would appear as a fragment of similar intensity to those in the rest of the profile. It is more likely that they resulted from incomplete digestion of the genomic DNA by the *Sal* I enzyme. The additional fragments are also not indicative of DNA degradation, because degradation is a random occurrence that would appear as a smear with no clearly visible fragments.

Table 4. Molecular weight estimates, in kilobases, of OpNPV fragments generated by digestion with *Sal* I restriction enzyme

Fragment	Estimated molecular weight	Reported molecular weight[a]
1	12.8	12.4
2	8.0	8.0
3	7.0	7.0
4	5.3	5.3
5	4.7	4.6
6	3.9	3.9
7	3.3	3.3
8	3.0	2.9
9	2.8	2.8
10	2.5	2.6
11	2.2	2.2
12	2.0	2.1
13	1.7	1.9
14	1.3	1.5
15	1.1	1.2
16	0.9	1.1
17	0.7	0.9
18	0.5	0.7

[a] Reported values taken from Miller and Dawes (1978) and Laitinen (1995).

Presence of OpCPV in TM BioControl-1®

A large, >23 kb band of OpNPV DNA was observed in each successful DNA extraction from the TM BioControl-1® samples. In TM BioControl-1® samples from lots 4a, 5b and 6, smaller weight bands were also observed in addition to this large band. These additional bands did not represent polymorphisms in the OpNPV genome, as they were observed in the final DNA extract before the OpNPV genome was digested. Further examination of these TM BioControl-1® samples indicated that in each sample there were 10 bands present ranging in size from 0.5 kb to 4.4 kb (Fig. 4A). The band profile closely matched that described for the double-stranded RNA virus, OpCPV (Laitinen et al. 1995) (Table 5). The molecular weight estimates of the lower weight segments did not match as closely with the reported values as did the higher weight segments. This discrepancy may be due to the use of different molecular standards for estimating fragment sizes. Molecular weights are estimated based on the mobility (distance traveled) of segments of known molecular weight. The mobilities of the standard fragments are plotted against their known molecular weights to generate a standard curve, and the mobilities of unknown segments are compared to this curve to estimate their molecular weights. Differences in the standards used to generate a standard curve may result in minor differences in molecular weight estimates.

Table 5. Molecular weight estimates, in kilobases, for putative OpCPV found in TM BioControl-1®

Segment	Estimated molecular weight	Reported molecular weight[a]
1	4.4	4.3
2	4.2	4.1
3	3.9	3.6
4	3.5	3.4
5	2.9	2.9
6	2.0	2.0
7	1.8	1.95
8	1.0	1.3
9	0.76	1.2
10	0.58	1.0

[a] Reported values taken from Laitinen et al. 1996.

To test whether the smaller bands present in the TM BioControl-1® samples were indeed RNA, replicate samples from lot 4a extract were treated with DNase I, RNase A, or with distilled water (Fig. 4B). DNase I is an enzyme that specifically digests DNA while RNase A is an enzyme that specifically digests RNA. In the replicate treated with DNase I, the smaller bands remained intact while the OpNPV DNA was digested. Conversely, in the replicate treated with RNase A, the smaller bands had disappeared while the OpNPV genomic DNA remained intact. The replicate treated with distilled water showed both the smaller bands and the OpNPV genomic DNA. The fact that RNase A digested the smaller bands while DNase I did not indicates that these bands were composed of RNA.

The electrophoretic profile of the RNA segments in samples of TM BioControl-1® from lots 4a, 5b and 6 indicated that these lots were contaminated with OpCPV. It is not known when, where, or how the CPV contamination was introduced. However, because not all of TM BioControl-1® samples contained OpCPV, it is likely that the MEM-75-STANDARD used to inoculate the *O. pseudotsugata* larvae for the original production of TM BioControl-1® was **not** the source of OpCPV. This is supported by the fact that the sample of MEM-75-STANDARD used as an experimental control in this study was also OpCPV free.

Discussion and Conclusion

OpNPV genomic DNA was successfully extracted from each of the 17 samples, one from each package size category of the 10 lots of TM BioControl-1®. Viral DNA extracted from each lot was consistently observed as a single, high molecular weight band larger than 23 kb (data not shown). Digestion of OpNPV genomic DNA with each of the restriction enzymes *Bgl* II, *Sal* I and *Pst* I, yielded predictable and repeatable restriction fragments. In every case, the samples of TM BioControl-1® displayed RFLP profiles that were consistent with the controls as well as with reported findings. These observations indicate that there is no discernible degradation and mutation, or genetic variation, of the OpNPV DNA in any of the lots of the stored TM BioControl-1®.

Degradation of DNA is a random occurrence, and if the viral DNA had been degraded the DNA extractions would have yielded fragments of many different sizes that would appear as a large smear with no resolvable fragments. This was not the case; for each sample, OpNPV DNA was observed as a single large band prior to REN digestion. DNA degradation would also be apparent in comparing the RFLP profiles of the samples. As degradation is a random event, it is unlikely that the DNA would degrade at the same rate or in the same manner from lot to lot. The DNA would still be digested by the restriction enzymes in areas where the DNA was still intact, but such variation among the samples of TM BioControl-1® would result in RFLP profiles that differed from the control and among the different lots. Due to the fact that no polymorphisms were detected in any of the stored TM BioControl-1® samples, it can be concluded that long-term storage of the product has not affected the structure of the viral genome. This is not surprising, as the virus contained in TM BioControl-1® is latent. With no chance to replicate, there is no opportunity for the genome to be altered by genetic mutation.

A contaminant was present in TM BioControl-1® samples from lot numbers 4a, 5b and 6. The electrophoretic profile and DNase I/RNase A treatments of this contaminant correlated with previous findings of Laitinen et al. (1995) and Galinski et al. (1982), and confirmed the identity of the contaminant to be OpCPV. This was not expected, although the presence of OpCPV in Douglas-fir tussock moth has been previously noted (Martignoni et al. 1969, Laitinen et al. 1996). It is unlikely that the presence of OpCPV adversely effects the potency of the product. Cytoplasmic polyhedroviruses differ from nucleopolyhedroviruses in that they produce a chronic rather than lethal pathenogensis in their host (Payne and Martens 1983). If anything, the presence of the OpCPV would be expected to enhance the pathogenicity of OpNPV, because the insects would have multiple infections to defend against rather than a single one. In fact, synergistic effects have been reported between NPV and CPV (Tanada 1956). However, further bioassays would be needed to confirm this assumption.

Fig. 1. RFLP profiles of 17 samples of stored TM BioControl-1® lots digested with *Bgl* II restriction endonuclease. Values above lanes indicate lot number of the samples. MEM denotes MEM-75-STANDARD DNA. DFTM indicates Douglas-fir tussock moth host DNA. H denotes Lambda *Hin*d III DNA standard. Numerical values on the left indicate the molecular sizes, in kilobases, of the Lambda *Hin*d III fragments.

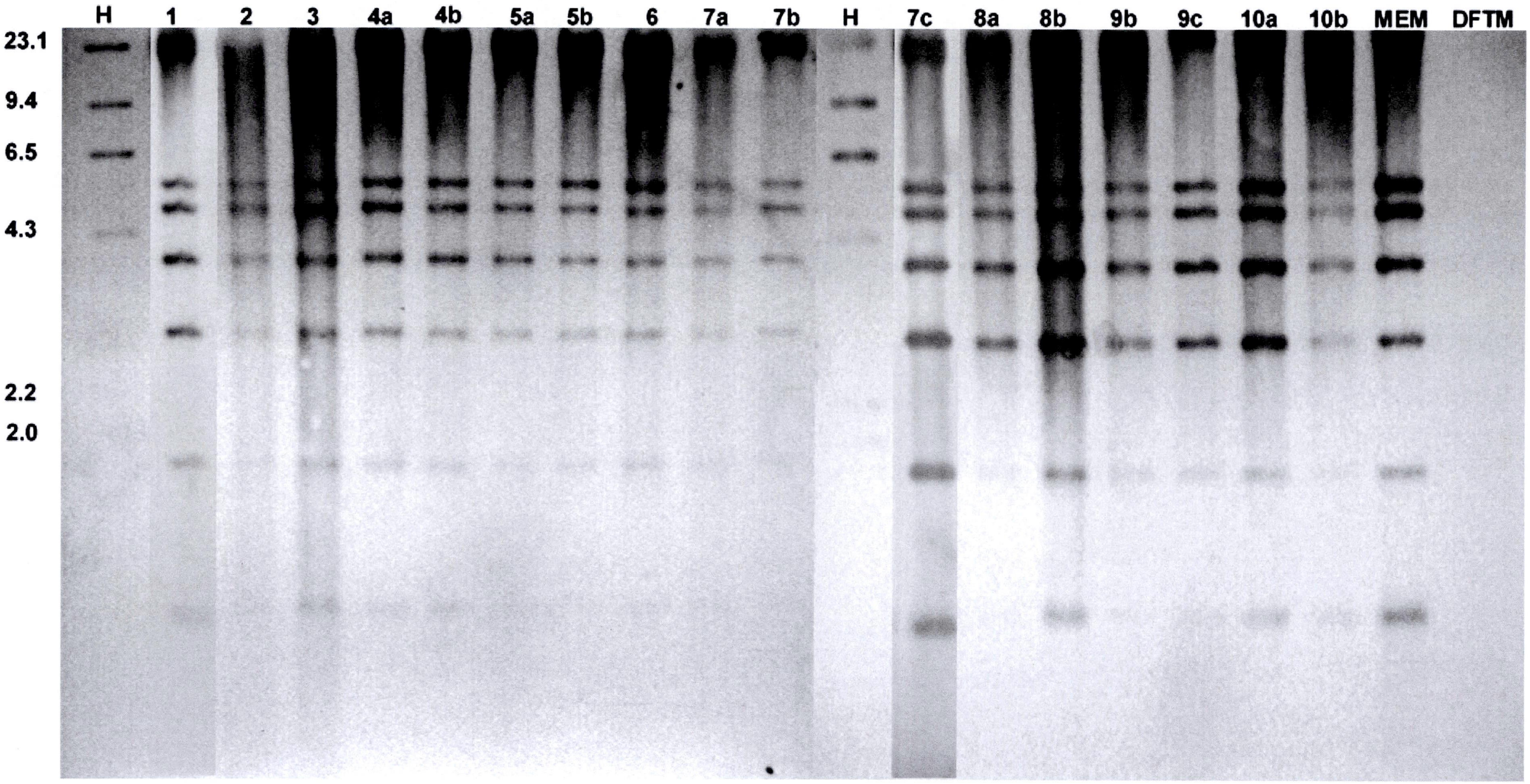

H
1
2
3
4a
4b
5a
5b
6
7a
7b
H
7c
8a
8b
9b
9c
10a
10b
MEM
DFTM
23.1
9.4
6.5
4.3
2.2
2.0

Fig. 2. RFLP profiles of 17 samples of stored TM BioControl-1® lots digested with *Pst* I restriction endonuclease. Values above lanes indicate lot number of the samples. MEM denotes MEM-75-STANDARD DNA. DFTM indicates Douglas-fir tussock moth host DNA. H denotes Lambda *Hin*d III DNA standard. Numerical values on the left indicate the molecular sizes, in kilobases, of the Lambda *Hin*d III fragments.

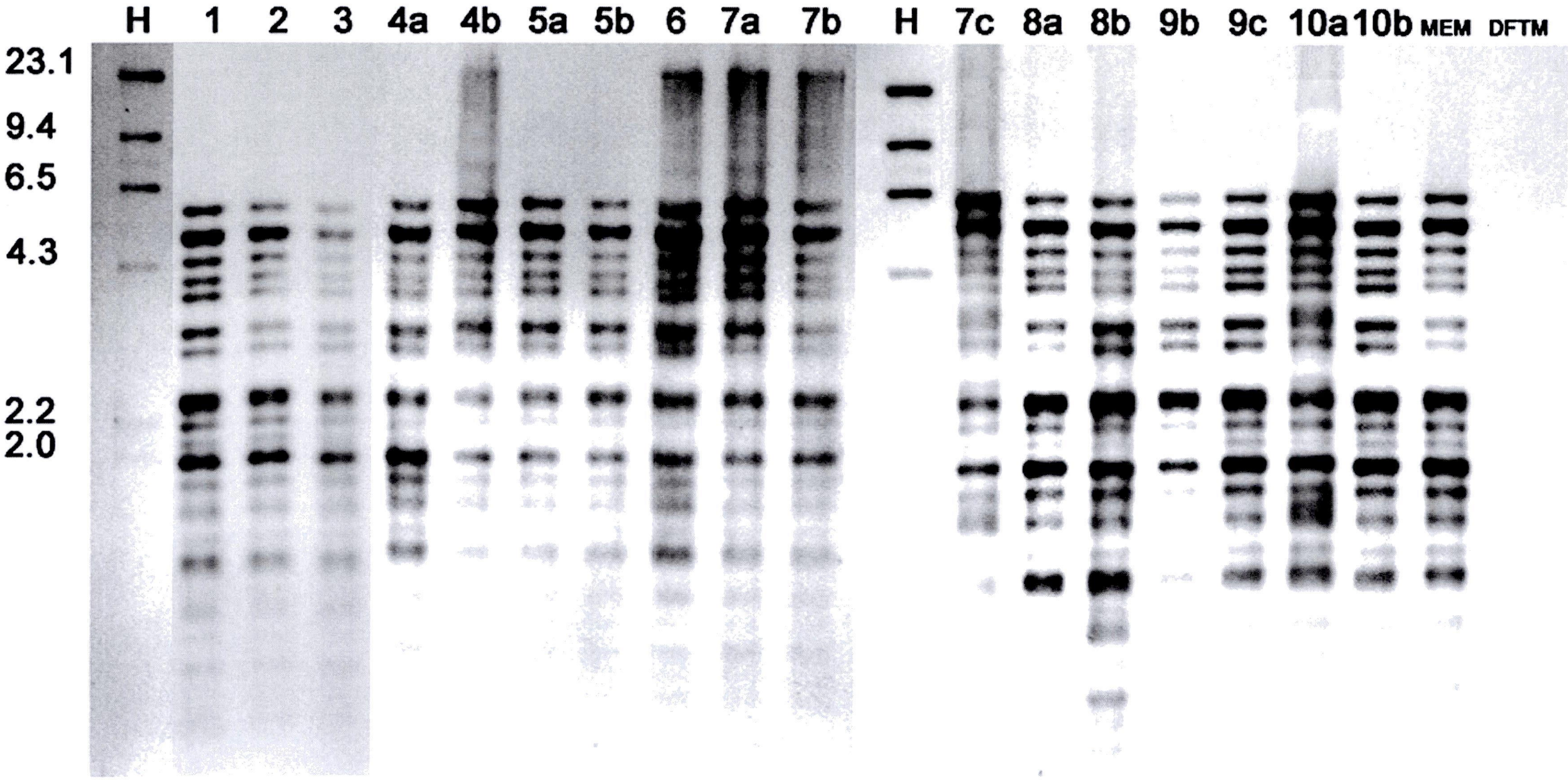

H
1
2
3
4a
4b
5a
5b
6
7a
7b
H
7c
8a
8b
9b
9c
10a
10b
MEM
DFTM
23.1
9.4
6.5
4.3
2.2
2.0

Fig. 3. RFLP profiles of 17 samples of stored TM BioControl-1® lots digested with *Sal* I restriction endonuclease. Values above lanes indicate lot number of the samples. MEM denotes MEM-75-STANDARD DNA. DFTM indicates Douglas-fir tussock moth host DNA. H indicates Lambda *Hin*d III DNA standard. Numerical values on the left indicate the molecular sizes, in kilobases, of the Lambda *Hin*d III fragments.

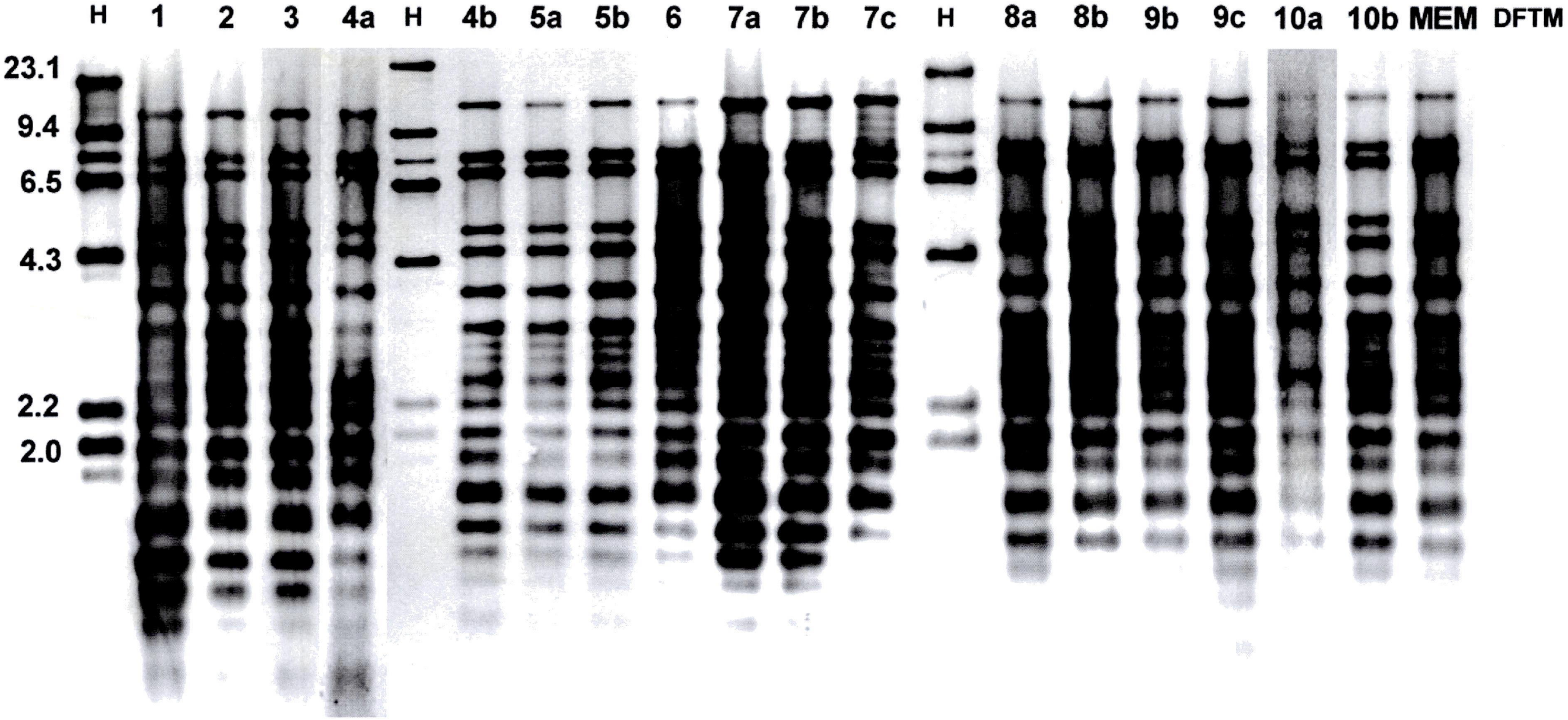
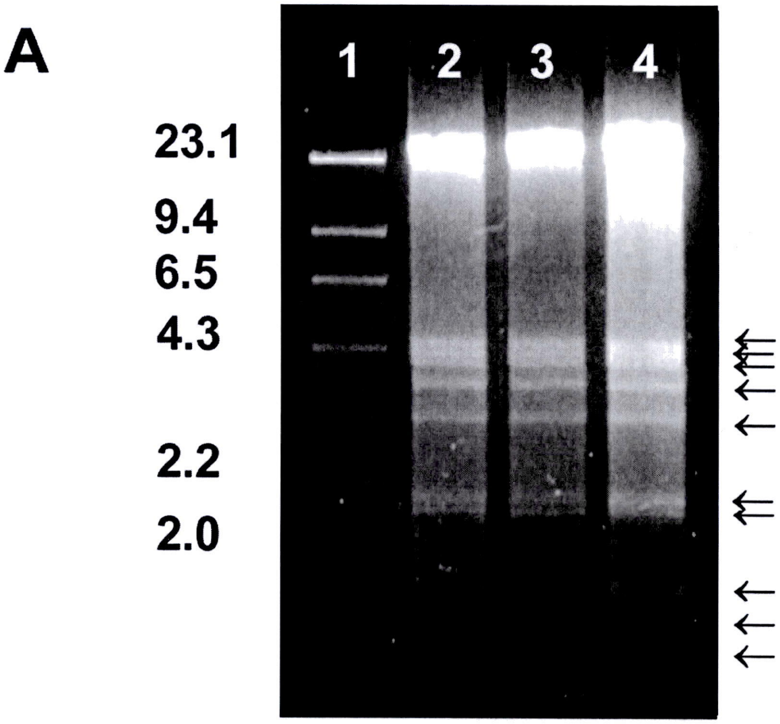
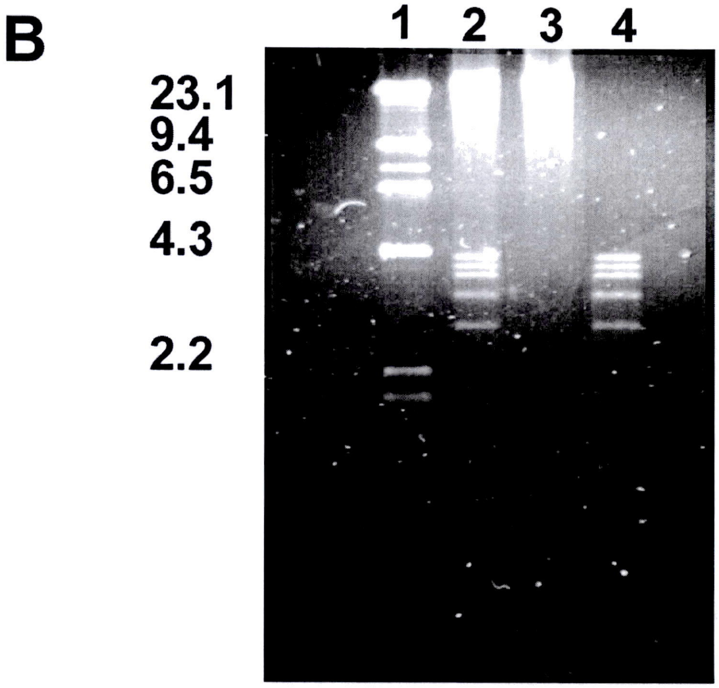

H
1
2
3
4a
H
4b
5a
5b
6
7a
7b
7c
H
8a
8b
9b
9c
10a
10b
MEM
DFTM
23.1
9.4
6.5
4.3
2.2
2.0

Fig. 4. A) OpNPV DNA extracts from samples of stored TM BioControl-1® from lot 4a (lane 2), lot 5b (lane 3) and lot 6 (lane 4) showing the presence of the contaminant, OpCPV. Numerical values on the left side indicate the molecular sizes of the Lambda *Hin*d III fragments (lane 1). Arrows on the right indicate the position of the 10 RNA segments of the OpCPV genome. B) RNase A and DNase I digestions of lot 4a OpNPV DNA extract. Lane 1: Lambda *Hin*d III standards. Numerical values on the left indicate size in kilobases of the segments. Lane 2: OpNPV DNA extract incubated in distilled water. Both OpNPV and OpCPV genomes remained intact. Lane 3: OpNPV DNA extract incubated with RNase A. Only the OpNPV genome remained intact. Lane 4: OpNPV DNA extract digested with DNase I. Only OpCPV genomic segments remained intact.


A
1
2
3
4
23.1
9.4
6.5
4.3
2.2
2.0


B
1
2
3
4
23.1
9.4
6.5
4.3
2.2

References Cited

Fox, J., Kossobudzki, S., and Fonseca da Cunha, J. 1943. Field studies on the immune response to 17-D yellow fever virus: relation to virus substrain, dose, and route of inoculation. Am. J. Hygiene 38: 113-138.

Galinski, M. S., K. Chow, G. Rohrmann, G. Pearson, and G. Beadreau. 1982. Size determination of *Orgyia pseudotsugata* cytoplasmic polyhedrosis virus. Virology 123: 328-335.

Hadfield, J. S., and R. W. Magelssen. 1995. TM BioControl-1: production history and inventory status. USDA Forest Service Region 6 Technical Bulletin, Corvallis, OR. 20 p.

Laitinen, A. M., I. S. Otvos, and D. B. Levin. 1996. Geographic distribution of cytoplamic polyhedrosis virus infection in Douglas-fir tussock moth larvae, *Orgyia pseudotsugata*, in British Columbia. J. Invertebr. Pathol. 67: 229-235.

Laitinen, A. 1995. Naturally–occurring nuclear polyhedrosis virus for the biological control of Douglas-fir tussock moth, *Orgyia pseudotsugata*, McDunnough, (Lepidoptera: Lymantridae) M.Sc. Thesis. University of Victoria, Victoria, BC.

Martignoni, M. E. 1999. History of TM BioControl-1: the first registered virus-based product for control of a forest insect. Am. Entomol. 45: 30-37.

Martignoni, M. E., P. J. Iwai, K. M. Hughes, and R. B. Addison. 1969. A cytoplasmic polyhedrosis virus of *Hemerocampa pseudotsugata*. J. Invertebr. Pathol. 13: 15-18.

Miller, L., and K. Dawes. 1978. Restriction endonuclease analysis for the identification of Baculovirus pesticides. Appl. Environ. Microbiol. 35: 411- 421.

Payne, C., and P. Mertens. 1983. The cytoplasmic polyhedrosis viruses, pp. 425-503. *In* W.K. Joklik, (ed.), The Reoviridae. Plenum Press, New York, NY.

Tanada, Y. 1956. Some factors affecting the susceptibility of the armyworm to virus infections. J. Econ. Entomol., 49: 52-57.

Thompson, C. G., and L. J. Peterson. 1978. Rearing the Douglas-fir tussock moth. USDA Agriculture Handbook No. 520, Combined Forestry Pest Research and Development Program, Washington, DC. 20p.

Section 6

Acre-Dose Calculations

B. Kukan and I.S. Otvos

Canadian Forest Service, Pacific Forestry Centre, Victoria, BC.

There are two ways to determine acre-dose of the stored TM Biocontrol-1® for use in the field to control Douglas-fir tussock moth outbreaks. The ideal way is through the use of two bioassays with the actual field populations of tussock moth that will be treated (Martignoni 1978, Scott 2000). The first or preliminary bioassay determines the potency and allows the selection of the appropriate virus concentration to use in the second bioassay. In the second bioassay the stored lot(s) of TM BioControl-1® to be used are tested against the field population to be treated. However, it is not always possible to do both of these bioassays due to time and funding constraints.

The second, less optimal method is to get a rough estimate of the acre-dose by referring to the activity standardization experiments done in this shelf-life determination project. We strongly emphasize that the first method is the proper way to determine the acre-dose for the stands to be protected. It is also by far the best, and the second method that we will describe here should only be used as a last resort.

When the TM BioControl-1® was first produced Martignoni and Iwai (1977, 1978) devised an activity standardization bioassay procedure to determine bioactivity titre. The Goose Lake laboratory strain was used in these bioassays testing the advance samples of TM BioControl-1® from the companies that processed the virus-killed cadavers. The acre-doses calculations are based on the recommended acre-dose of TM BioControl-1® of 1.085×10^9 AU_{GL}, where AU_{GL} is the activity unit based on the response of the Goose Lake strain. Each of the lots of TM BioControl-1® will have a potency or activity titre that equates to an acre-dose of treatment expressed as the number of activity units per gram of preparation (Martignoni 1978). PIBs/ng for each lot, of TM BioControl-1® produced, was determined at the time of production.

The acre-dose was calculated from the LD_{50} values of the bioassays, done for each of the lots in this shelf-life determination project, in the following way:

LD_{50} in ng = 1 AU (activity unit)

changed this to AU/g : $1 \times 10^9 \div LD_{50}$ in ng = $? \times 10^9$ AU/g (this is the potency)

Using the recommended acre-dose of 1.085×10^9 AU/acre calculate the g/acre:

1.085×10^9 AU / acre $\div\ ? \times 10^9$ AU/g = # g/acre

The new or current acre-dose values, calculated from the LD_{50} values obtained by the bioassays conducted between 1998 and 2001 in the shelf-life determination project, are presented in Table 1. We recommend to use the least potent lots first, i.e. lots 1, 3, 4, 10, 5, 2, etc. This would allow keeping the more potent lots (lots 7, 6, 9 and 8) for longer, and even with the expected degradation of the efficacy, these four lots are likely to remain effective to control Douglas-fir tussock moth outbreaks into the 2020s.

It is recommended that, ideally, the stored lots of TM-Biocontrol-1® (kept at –10°C) should be re-bioassayed every 15-20 years to determine if further degradation of the product has occurred and re-calculate the acre-dose as required. Ideally, each unused lot should be bioassayed with three replicates. A note of caution, if the lots are not re-tested and the acre-dose values to be used during the next outbreak in the 2010s are based on "best estimates" (Table 1) without any further bioassays, the acre-dose estimates given in Table 1 may or may not provide adequate foliage protection in the suppression project.

Table 1. Summary of acre-dose values calculated for the 10 lots of TM BioControl-1® in storage[a], using median lethal dose values from bioassays conducted between 1998 and 2001 with third instar Goose Lake Douglas-fir tussock moth larvae

Lot #	PIBs/ng	LD_{50} PIBs	LD_{50} ng	AU/g	g/acre
1	13.5	77.1	5.7	0.18×10^9	6.0
2	15.1	49.6	3.3	0.30×10^9	3.6
3	12.9	60.7	4.7	0.21×10^9	5.2
4	12.8	52.1	4.1	0.24×10^9	4.5
5	12.8	45.7	3.6	0.28×10^9	3.9
6	29.6	44.5	1.5	0.67×10^9	1.6
7	41.5	46.1	1.1	0.91×10^9	1.2
8	42.5	113.8	2.7	0.37×10^9	2.9
9	38.0	55.9	1.5	0.67×10^9	1.6
10	14.5	54.0	3.7	0.27×10^9	4.4

[a] TM BioControl-1® product is currently in storage at –10°C at the USDA Forest Service facility in Corvallis, OR.

References Cited

Martignoni, M. E. 1978. Virus in biological control: production, activity, and safety, pp. 140-147. *In* M. H. Brookes, R. W. Stark, and R. W. Campbell (eds.), The Douglas-fir tussock moth: a synthesis. USDA Technical Bulletin 1585, USDA Forest Service, Science and Education Agency, Washington, DC.

Martignoni, M. E., and P. J. Iwai. 1977. Peroral bioassay of technical-grade preparations of the Douglas-fir tussock moth nucleopolyhedrosis virus (Baculovirus). USDA Forest Service Research Paper PNW-222, Pacific Northwest Forest and Range Experiment Station, Portland, OR. 12 p.

Martignoni, M. E., and P. J. Iwai. 1978. Activity standardization of technical preparations of Douglas-fir tussock moth *Baculovirus*. J. Econ. Entomol. 71: 473-476.

Scott, D. 2000. Activity standardization and determination of acre dose of TM BioControl-1® preparation. Internal USDA Forest Service document.

Section 7

Incidence of egg parasitism, level of naturally occurring virus, and egg viability in field collected Douglas-fir tussock moth egg masses from California, Oregon, Idaho, and Washington, 1998-2001

I.S. Otvos, and N. Conder

Canadian Forest Service, Pacific Forestry Centre, Victoria, BC.

Introduction

Several natural mortality factors influence generation survival of the Douglas-fir tussock moth, including parasitism, predation and diseases. Of these mortality agents, a nucleopolyhedrovirus is most frequently associated with the collapse of outbreaks. Parasitoid and predator complexes can (to a lesser degree) also influence the population dynamics of the Douglas-fir tussock moth. The role of parasitoids and diseases is known to increase with the progression of defoliator outbreak. Although Torgersen and Ryan (1981) reported ca. 50% egg parasitism by *Telenomus californicus* in the spring, in both planted and naturally laid egg masses, generally this level of parasitism is not considered high enough to bring about the collapse of the outbreak. Such high levels of egg parasitism will have an important negative impact on the host population, but it is likely to occur or be detected too late to influence control decisions. However, it may be still worth investigating the role of *Telenomus californicus* in the population dynamics of Douglas-fir tussock moth, because a complex of species belonging to the same genus have been credited with the collapse of a large outbreak of another forest defoliator, the eastern hemlock looper, *Lambdina fiscellaria fiscellaria* (Hébert et al. 2001).

Steinhaus (1951) reported a nucleopolyhedrosis for the first time in the Douglas-fir tussock moth from Washington, Oregon and Idaho. Since that time this virus has been reported to cause population collapses in British Columbia (Sudgen 1957, Morris 1963), Washington, Idaho and Montana (Tunnock 1973), Oregon and California (Wickman et al. 1973), and California (Dahlsten and Thomas 1969, Mason and Thompson 1971).

Surface contamination of eggs by naturally occurring virus is well established in the Douglas-fir tussock moth (Thompson 1978), as well as in other insects (Bergold 1942, Thompson and Steinhaus 1950, Leonard and Doane 1966). Presence of NPV can be detected in the beginning of the year in which the virus epizootic first develops (Thompson 1978). The presence of NPV-contaminated eggs is so consistent that attempts have been made to use it to predict impending epizootic and subsequent collapse of outbreaks (Thompson 1978).

It has been suggested that when percent infection by naturally occurring virus, determined from larval rearing from field collected egg masses, reaches the 20-30% range Douglas-fir tussock moth populations are very likely to collapse and suppression of the population is not required.

Although the work described in this section was not part of the cooperative agreement, we volunteered to do it to see if we could improve on the accuracy of the broad threshold level. We volunteered to rear egg masses to try to determine if an accurate or more refined “threshold level” can be established for the level of naturally occurring virus and egg parasitism above which there would be no need to treat because the outbreak would collapse on its own. Through the rearing of field collected eggs in the laboratory one can determine the health of Douglas-fir tussock moth populations and can make predictions on the course of the outbreak.

We reared Douglas-fir tussock moth egg masses collected from different locations in California, Oregon, Idaho, and Washington over the last 3 years. The results of these rearings are summarized in this section.

Materials and Methods

Ideally, 50 egg masses are considered to "represent" a site biologically, but 25-30 egg masses from an area may be enough to give an indication of what is likely to happen to the Douglas-fir tussock moth population in that area. Douglas-fir tussock moth egg masses were collected at five sites in Sequoia and Kings Canyon National Parks in California in the fall of 1998. In 1999, egg masses were collected at four sites in Oregon and 11 sites in Idaho. However, eggs from only four sites in Idaho were reared because the number of egg masses collected at the other sites was too low (1-8) to give meaningful results. In 2000, egg masses were collected at two sites in Washington, nine sites in Oregon and four sites in Idaho.

Depending on egg mass density, up to 50 new egg masses were collected at each site and placed individually in small paper bags, and these from each site were placed in a larger paper bag and labelled by collection site and collection dates. The egg mass from each State was sent by courier service to Pacific Forestry Centre (Natural Resources Canada, Canadian Forest Service) in Victoria, BC. In Victoria the egg masses were stored at 4-5°C for 4-6 months to break diapause. In early to mid-April, after the diapause requirement was satisfied, the egg masses were moved to the laboratory where they were gently teased apart and about 40 eggs from each egg mass were reared individually in 96-well ELISA plates by location (the rest of the eggs from the egg masses were used in bioassays to determine efficacy of the stored product or the effect of carrier 038a® on the feeding behavior of the Douglas-fir tussock moth larvae). The eggs were reared individually to prevent re-parasitization by *Trichogramma* sp., probably *minutum*, as this species emerges about 4-6 days earlier than the host and can oviposit and develop in the unparasitized host eggs. *Telenomus* sp, probably *californicus*, is another egg parasitoid, and it emerges about 5-10 days after host larval emergence is completed and only a single specimen emerges from a host egg.

The eggs were reared at 25±1°C, 40-50% RH and 12:12 (L:D). Eggs were reared until the emergence of host larvae or parasitoid emergence was completed (approximately 35 days). Level of the naturally occurring NPV infection was determined by using the method of Stelzer (1979) and egg parasitism by species was determined by identifying emerged parasitoids using the taxonomic key given in Torgersen and Mason (1979).

In most cases more than 25 larvae emerged from the sub-sample of eggs separated from each egg mass. Twenty-five newly emerged larvae were placed in 15 x 100 mm petri dishes with a 2 x 2 cm square piece of tussock moth diet (Thompson and Peterson 1978) and reared at 25±1°C, 40-50% RH and 12:12 (L:D). The diet was changed after 7 days and the dead larvae removed. At day 14 the rearing was stopped and the additional dead larvae were also removed. Smears were made of all the larvae (after 1999) that died by day 14, examined under a compound microscope, and scored for the presence of NPV (Stelzer 1979).

Results

1998-1999 Generation

About 40-50 eggs from each of the 245 egg masses collected at five sites in Sequoia and Kings Canyon National Parks (a total of 9,800 eggs) were reared. No egg parasitoids emerged from the 9,800 eggs reared from California. Percent larval emergence was not determined, and of the 3,990 larvae reared for virus determination, 1,719 larvae died. Because of the high number of dead larvae only about 40% of them were diagnosed under a compound microscope for presence of virus.

The level of viral infection varied among the five sites between 16.4 and 33.5% averaging 21.9% for the outbreak in Sequoia and Kings Canyon National Parks (Table 1). Based on this average infection of 21.9% it was predicted that the Douglas-fir tussock moth outbreak in California was likely to collapse in 1999, but not necessarily before visible defoliation/damage would occur.

Based on many considerations, including this relatively high incidence of natural viral infection determined from egg masses, the USDA Forest Service did not treat the Douglas-fir tussock moth outbreak in California. The outbreak did collapse and when the area was later visited during larval surveys many tussock moth larvae were found hanging from the branches (typical for virus killed larvae) (J. Wenz, personal communication). During these larval surveys, a sub-sample of the hanging larvae were collected, up to 10 larvae at each location, and were sent to Victoria for diagnosis. In Victoria all the dead larvae were examined, with the aid of a compound microscope, for the presence of NPV. The results of this diagnosis are presented in Table 2. Because only up to 10 larvae per location per collection date were sent for diagnosis the results (percent viral infection) based on these low numbers should be used with caution. No viral infection was detected among the dead larvae in the first collection and we have no explanation for this. We can only say that the level of virus infection increased with each successive sample date, as expected. The virus epizootic must have been thorough, because even the cadavers were difficult to find as indicated by the fewer than 10 larvae that could be collected at some of the sample locations and dates. Besides NPV PIBs a second type of occlusion bodies was also found in some of the cadavers. We hope to conduct DNA analysis to determine if these larvae had a mixed viral infection or if the second type of occlusion bodies represents only contamination.

Table 1. Percent parasitism and level of naturally-occurring virus infection from Douglas-fir tussock moth egg masses collected in Sequoia and Kings Canyon National Parks, California in January 1999 and determined in Victoria, BC, April 1999

Coding	Location	No. egg masses reared	Total no. eggs reared[a]	Total no. larvae emerged	Percent larval emergence	No. eggs parasitized by Trich.[b]	Percent parasitism by Trich.[b]	No. eggs parasitized by Tele.[c]	Percent parasitism by Tele.[c]	**Total % Parasitism**	Total No. larvae reared	No. Missing[d]	No. Dead Larvae[e]	% Larval Mortality	No. Dead Larvae with NPV	% Dead Larvae with NPV[f]	**Est. % NPV Infection[g]**
1 - 50	Site 1	50	2000	-	-	0	0.0	0	0.0	**0.0**	855	64	260	32.9	68	52.3	**17.2**
51 - 100	Site 2	47	1880	-	-	0	0.0	0	0.0	**0.0**	537	45	191	38.8	39	47.0	**18.2**
101 - 150	Site 3	49	1960	-	-	0	0.0	0	0.0	**0.0**	877	34	355	42.1	56	38.9	**16.4**
151 - 200	Site 4	50	2000	-	-	0	0.0	0	0.0	**0.0**	844	60	455	58.0	64	39.3	**22.8**
201 - 250	Site 5	49	1960	-	-	0	0.0	0	0.0	**0.0**	877	70	458	56.8	98	59.0	**33.5**
Total	-	245	9800	-	-	0	0.0	0	0.0	**0.0**	3990	273	1719	46.2	325	47.4	**21.9**

[a] About 40 eggs per egg mass were reared. However, if more than 25 larvae emerged from the eggs the extra larvae were not counted. Therefore, total larval emergence cannot be calculated.

[b] *Trichogramma minutum* complex

[c] *Telenomus californicum*

[d] Sum of number of larvae killed by handling and number of larvae unaccounted for after assay was completed.

[e] Smears were made of 686 (39.9%) dead larvae for virus determination.

[f] Value calculated by formula (No. smears with NPV/No. dead larvae examined) x 100

[g] Value calculated by formula (**[** { No. smears with NPV/No. dead larvae examined } x No. dead larvae **]** / No. larvae reared) x 100

Table 2. Occurrence of virus over time in dead Douglas-fir tussock moth larvae collected in Sequoia and Kings Canyon National Parks, 1999.

	% larvae infected by NPV[a]				
Location/Date collected	July 15	July 20	Aug. 11	Aug. 24	Average[b]
A- Grant Grove Stables	0.0	0.0	40.0	40.0	19.5 (25.8)
B- Crystal Springs Campground	0.0	10.0	0.0	0.0 [1]	3.2 (4.8)
C- Quait Flat	0.0	10.0	22.2 [9]	0.0 [6]	8.6 (12.0)
D- Sunset Campground	0.0	0.0	50.0	62.5 [8]	26.3 (35.7)
E- Dorset Campground	0.0	10.0	100.0 [2]	-	13.6 (25.0)
F- Beetle Rock	0.0	0.0	10.0	0.0 [8]	2.6 (3.6)
G- Swale	-[c]	66.7 [3]	-	-	66.7 (66.7)
Average	0.0	7.8	27.5	27.3	13.5 (18.9)

[a] Number of dead larvae examined for NPV given in square brackets if < 10.
[b] Percentages in brackets calculated without first collection.
[c] No cadavers received for diagnosis.

1999-2000 Generation

Of the 60 egg masses collected at four locations in Oregon and 48 egg masses at four locations in Idaho in the fall of 1999, 40 eggs were reared from each of these egg masses. The results of these rearings are given in Table 3. In Oregon, larval hatch varied from about 71 to 89% among the four locations. Egg parasitism was less than 1% at one and 0% at the other three locations. Level of viral infection was also low, 0% at Pine Rd., and less than 1% at two other sites (Pine Rd. East and Wallow-Whitman). At the fourth location, Fish Creek, 11 of the 12 dead larvae with NPV came from one egg mass.

All three parameters, high larval hatch, low parasitism, and low incidence of naturally occurring virus, indicated a vigorous Douglas-fir tussock moth population in

Oregon that was predicted to increase in size and damage intensity in 2000 without suppression.

Rearing of eggs from Idaho showed similar results. Larval hatch ranged between 85 and 94%, averaging 89% for the four locations examined (Table 3). Egg parasitism was very low (<1%) and no viral infection was detected among the larvae reared (Table 3).

The same three parameters, high larval hatch, very low egg parasitism, and no presence of the naturally occurring virus, indicated an even healthier (younger) population in Idaho than in Oregon. It was predicted that the Douglas-fir tussock moth population in Idaho would also increase both in size and its damage intensity.

2000-2001 Generation

In the fall of 2000, Douglas-fir tussock moth egg masses were collected in Oregon (23), Idaho (145) and Washington (45) (Table 4). Rearing of eggs from these egg masses showed reduced larval hatch and continued low parasitism in all three States. In Oregon, larval hatch was 65% and 81% for the two locations, averaging 76% for the State. Egg parasitism was <1% and only *Telenomus californicus* was recovered. Level of larval infection was also low averaging about 1% for this State. The three parameters indicate that the Douglas-fir tussock moth outbreak in Oregon should peak in 2001 and some expansion of the outbreak is likely to occur but overall damage intensity (defoliation) should be less than it was in 1999.

In Idaho, larval hatch ranged from 81% to 93% among the four locations, averaging 82% for the State. Total combined egg parasitism was <1% even though both *Trichograma minutum* and *Telenomus californicus* occurred. Level of naturally occurring virus was low, <1%. The three parameters, high larval emergence, low egg parasitism, and low incidence of virus suggest increasing population, increase in the size of outbreak, and increase in the intensity of defoliation.

In Washington, larval emergence was 89% and 78% at the two locations, averaging 82% for the State. The average total egg parasitism was 5.4%. *Trichograma minutum* made up 0.7% and the remaining 4.8% was contributed by *Telenomus californicus*. The most interesting part was the relatively high incidence of the naturally occurring virus, 17% at Yellow Jacket and about 13% at the Early Winter site. Based on the values of the three parameters it is predicted that the outbreak should peak in 2001 and decline in 2002. It would be most interesting to collect egg masses for rearing to determine how these parameters will change in 2002.

Table 4. Douglas-fir tussock moth larval emergence, percent parasitism, and level of naturally-occurring virus infection from egg masses collected in Washington, Oregon, and Idaho in the fall of 2000 and determined in Victoria, BC, April 2001

OREGON

Coding	Location	No. egg masses reared	Total no. eggs reared[a]	Total no. larvae emerged	Percent larval emergence	No. eggs parasitized by Trich.[b]	Percent parasitism by Trich.[b]	No. eggs parasitized by Tele.[c]	Percent parasitism by Tele.[c]	**Total % Parasitism**	Total No. larvae reared	No. Missing[d]	No. Dead Larvae[e]	% Larval Mortality	No. Dead Larvae with NPV	% Dead Larvae with NPV[f]	**Est. % NPV Infection[g]**
Jub	Jubilee	7	280	182	65.0	0	0.0	1	0.4	**0.4**	154	0	17	11.0	2	11.8	**1.3**
OR	Misc. Oregon	16	640	519	81.1	0	0.0	0	0.0	**0.0**	371	2	21	5.7	2	9.5	**0.5**
Total	Total Oregon	23	920	701	76.2	0	0.0	1	0.1	**0.1**	525	2	38	7.3	4	10.5	**0.8**

IDAHO

Coding	Location	No. egg masses reared	Total no. eggs reared[a]	Total no. larvae emerged	Percent larval emergence	No. eggs parasitized by Trich.[b]	Percent parasitism by Trich.[b]	No. eggs parasitized by Tele.[c]	Percent parasitism by Tele.[c]	**Total % Parasitism**	Total No. larvae reared	No. Missing[d]	No. Dead Larvae[e]	% Larval Mortality	No. Dead Larvae with NPV	% Dead Larvae with NPV[f]	**Est. % NPV Infection[g]**
MSC	Moscow Mtn.	49	1960	1582	80.7	1	0.1	15	0.8	**0.8**	1165	7	80	6.9	1	1.3	**0.1**
SD	Skyline Drive	46	1840	1616	87.8	1	0.1	3	0.2	**0.2**	1143	4	125	11.0	10	8.0	**0.9**
	Subtotal	95	3800	3198	84.2	2	0.1	18	0.5	**0.5**	2308	11	205	8.9	11	5.4	**0.5**
GH	Gold Hill	26	1040	967	93.0	1	0.1	0	0.0	**0.1**	650	14	36	5.7	2	5.6	**0.3**
MM	Moses Mtn.	24	960	859	89.5	0	0.0	1	0.1	**0.1**	600	12	45	7.7	0	0.0	**0.0**
	Subtotal	50	2000	1826	91.3	1	0.1	1	0.1	**0.1**	1250	26	81	6.6	2	2.5	**0.2**
Total Idaho		145	5800	5024	82.4	3	0.7	19	0.3	**0.4**	3558	37	286	8.1	13	4.5	**0.4**

WASHINGTON

Coding	Location	No. egg masses reared	Total no. eggs reared[a]	Total no. larvae emerged	Percent larval emergence	No. eggs parasitized by Trich.[b]	Percent parasitism by Trich.[b]	No. eggs parasitized by Tele.[c]	Percent parasitism by Tele.[c]	**Total % Parasitism**	Total No. larvae reared	No. Missing[d]	No. Dead Larvae[e]	% Larval Mortality	No. Dead Larvae with NPV	% Dead Larvae with NPV[f]	**Est. % NPV Infection[g]**
OYJ	Yellow Jacket	17	680	608	89.4	0	0.0	39	5.7	**5.7**	421	82	79	23.3	59	74.7	**17.4**
OEW	Early Winters	28	1120	875	78.1	12	1.1	47	4.2	**5.3**	646	53	132	22.3	75	56.8	**12.6**
Total	Total Washington	45	1800	1483	82.4	12	0.7	86	4.8	**5.4**	1067	135	211	23.1	134	63.5	**14.4**

[a] About 40 eggs per egg mass were reared. The remaining eggs were kept for the bioassaying for the effects of carrier 038® on foliage.

[b] *Trichogramma minutum* complex

[c] *Telenomus californicum*

[d] Sum of number of larvae killed by handling and number of larvae unaccounted for after assay was completed.

[e] Smears were made of all dead larvae for virus determination.

[f] Value calculated by formula (No. smears with NPV/ No. dead larvae examined) x 100

[g] Value calculated by formula (No. smears with NPV/ No. larvae examined) x 100

Recommendations

Rearing can only be done after the diapause requirement is satisfied by cold storage, a minimum of 3-4 months. Generally, the results of larval hatch, egg parasitism, and level of the naturally occurring virus cannot be summarized much earlier than April to mid-May, because of the cold storage requirement plus 3-4 weeks of rearing and diagnosis. Forest managers faced with making a control decision would like to have these data earlier to have more lead-time for preparation of a suppression project including letting of contract for spray aircrafts. More lead-time may be achieved by collecting egg masses from mid-September through November and placing them in cold storage earlier. That would move the whole process forward and the rearing results may be available in March the following year.

It may also be worthwhile to collect egg masses twice from the same general location: once in the fall and then again in late spring (late April). Larval hatch, egg parasitism and level of natural virus infection from these two collections of egg masses could then be compared.

Torgersen and Ryan (1981) reported considerable increase in percent parasitism by *Telenomus californicus* between fall and spring collected egg masses. Collecting egg masses in late April to get the maximum parasitization would leave no, or very little, time for planning the suppression project. Further studies on egg parasitism by *T. californicus* in the spring may indicate a greater role in the collapse of its host population than currently thought. *Telenomus* spp. have been credited with the collapse of a large eastern hemlock looper outbreak in Quebec (Hébert et al. 2001).

While the rearings of the spring collected egg masses may not provide the lead time desired by forest managers responsible for making control decisions, it may provide useful additional biological and ecological data on egg viability, level of viral infection and egg parasitism.

References Cited

Bergold, G. H. 1942. Polyederfreie Insektenzuchten. Die Naturwiss. 30: 422-424.

Dahlsten, D. L., and G. M. Thomas. 1969. A nucleopolyhedrosis virus in populations of the Douglas-fir tussock moth, *Hemerocampa pseudotsugata*, in California. J. Invert. Pathol. 13: 264-271.

Doane, C. C. 1970. Primary pathogens and their role in the development of an epizootic in the gypsy moth. J. Invertebr. Pathol. 15: 21-33.

Doane, C. C. 1975. Infectious sources of nuclear polyhedrosis virus persisting in natural habitats of the gypsy moth. Environ. Entomol. 4: 392-394.

Hébert, C., R. Berthiaume, A. Dupont, and M. Auger. 2001. Population collapses in a forecasted outbreak of *Lambdina fiscellaria* (Lepidoptera: Geometridae) caused by spring egg parasitism by *Telenomus* spp. (Hymenoptera: Scelionidae). Env. Entomol. 30(1): 37-43.

Leonard, D. E., and C. C. Doane. 1966. An artificial diet for the gypsy moth, *Porthetria dispar*. Ann. Entomol. Soc. Am. 59: 462-464.

Mason, R. R., and C. G. Thompson. 1971. Collapse of an outbreak population of the Douglas-fir tussock moth, *Hemerocampa pseudotsugata* (Lepidoptera: Lymantriidae). USDA For. Serv. Res. Note PNW-139, Pac. Northwest For. and Range Exp. Stn., Portland, OR, 9 pp.

Morris, O. N. 1963. The natural and artificial control of the Douglas-fir tussock moth, *Orgyia pseudotsugata* McDunnough, by a nuclear polyhedrosis virus. J. Insect Pathol. 5(4): 401-414.

Steinhaus, E. A. 1951. Report on diagnosis of diseased insects 1944-1950. Hilgardia 20(22): 629-678.

Stelzer, M. J. 1979. How to determine the occurrence of virus in egg masses. USDA Agriculture Handbook No. 548, 7 pp.

Sudgen, B. A. 1957. A brief history of outbreaks of Douglas-fir tussock moth, *Hemerocampa pseudotsugata* McD., in British Columbia. Entomol. Soc. B.C. Proc. 54: 37-39.

Thompson, C. G. 1978. Nuclear polyhedrosis epizootiology, pp. 136-139. *In* M. H. Brookes, R. W. Stark and R. W. Campbell (eds.), The Douglas-fir tussock moth: a synthesis. Technical Bulletin 1585, USDA Forest Service, Science and Education Agency, Washington, DC.

Thompson, C. G., and L. J. Peterson. 1978. Rearing Douglas-fir tussock moth. USDA Agriculture Handbook No. 520, 7 pp.

Thompson, C. G., and E. A. Steinhaus. 1950. Further tests using a polyhedrosis virus to control the alfalfa caterpillar. Hilgardia 19(14): 411-445.

Torgersen, T. R., and R. R. Mason. 1979. Predation and parasitization of Douglas-fir tussock moth egg masses. USDA Agriculture Handbook No. 549, 11 pp.

Torgersen, T. R., and R. B. Ryan. 1981. Field biology of *Telenomus californicus* Ashmead, an important egg parasite of Douglas-fir tussock moth. Ann. Entomol. Soc. Am. 74(2): 185-186.

Tunnock, S. 1973. The Douglas-fir tussock moth in the northern region- a cartographic history of the outbreaks from 1928-1973. USDA Forest Service, Div. State and Priv. For., Rep. 73-27, Missoula, MT, 18 pp.

Wickman, B.E., R. R. Mason, and C. G. Thompson. 1973. Major outbreaks of the Douglas-fir tussock moth in Oregon and California. USDA Forest Service General Technical Report PNW-5, Pacific Northwest Forest and Range Experimental Station, Portland, OR, 18 pp.

Section 8: Conclusions and Recommendations

I. S. Otvos, Canadian Forest Service, Pacific Forestry Centre, Victoria, BC.

Conclusions

1) The stored TM BioControl-1® lots are still effective against the Douglas-fir tussock moth even with their ca. 30% decrease in efficacy. There is enough of this product stored for suppression of Douglas-fir tussock moth during the current and next outbreak and perhaps beyond. TM BioControl-1® lots with lower efficacy (those with higher acre-dose requirements) should be used first either in the remaining years of the current outbreak or during the next outbreak.
2) Carrier 038a® does not have anti-feeding properties and it may even have some feeding stimulant effects. Therefore, its use as a carrier to deliver TM BioControl-1® to control Douglas-fir tussock moth outbreaks is appropriate.
3) As expected, third instar larvae need more, about four times more, PIBs to kill 50% of the larvae than in the second instar. Using the diet surface contamination method it takes about two times the amount of virus to kill 50% of the second instar larvae than with the diet plug method.
4) Feeding on Douglas-fir foliage after infection does not appear to influence the infection process.
5) Storing TM BioControl-1® product at –10°C for 5-15 years did not affect or change the genomic structure of the virus in TM BioControl-1®.
6) The new acre-dose values of the various lots of TM BioControl-1® after 5-15 years of storage are presented in Table 1.
7) Egg viability, percent egg parasitism and level of naturally occurring virus may be used to predict the course of outbreaks, but the threshold levels indicating this need refinement.

Recommendations

1) Consider testing the efficacy of TM BioControl-1® at reduced dosages in the United States to suppress Douglas-fir tussock moth outbreaks, similar to the work done on this in Canada. This should only be tested in "new" outbreak areas where the incidence of natural virus infection is low. If acceptable suppression is achieved with reduced dosages, using TM BioControl-1® at reduced dosages will "increase the apparent acre-dose" of the remaining lots.
2) a) Try to determine a more accurate threshold level, that will indicate that the outbreak is collapsing and no suppression project is needed, than the currently used 20-30% infection by the naturally occurring virus. Refinement of this may be achieved during the current outbreak.

   b) The changes in percent egg parasitism, larval hatch and level of natural virus incidence should be explored, both within a generation (fall and spring collected egg masses), and between generations (during an outbreak cycle) of the Douglas-fir tussock moth.
3) The virus detection kit for field use should be completed, tested, and its potential for use should be explored in the field during the current outbreak.
4) The development of a sequential egg-mass survey (perhaps similar to that developed in BC for Douglas-fir, one of the host trees) is also needed in the US. The pheromone monitoring system, which seems to be working well for the Douglas-fir tussock moth in the US, acts as a warning, and indicates developing outbreaks. However, it is generally accepted that the next stages of sampling (both egg-mass and larval surveys) and the currently used sampling intensity cannot refine these broad predictions sufficiently enough to identify threatened stands requiring control measures. The sequential egg-mass survey method, used in BC, should be tested

on other host trees in the US. Based on the results of these tests the BC method may need to be adapted for use on different host trees, or perhaps require the development of a completely new sequential egg-mass survey method in the US. Work on this sequential egg-mass survey probably should only be started during the next outbreak when the outbreak is developing so that expected damage/defoliation can be predicted from egg-mass densities in a new, building population.

8) It will be highly desirable to do a few preliminary tests to confirm the efficacy of TM BioControl-1® lots in storage at the start of the next outbreak to determine if re-testing all the remaining lots of the product is needed.
9) It may be worth investigating genotypic variation of the Douglas-fir tussock moth virus in the western US, and whether or not the virus occurs in Arizona (southern end of distribution) and compare these with those in BC (northern end of distribution).

Recommendation numbers 1, 2a, 2b and 3 can still be done during the current outbreak. Number 4 may be initiated during the remaining years of this outbreak, but the greatest value of the sequential egg-mass survey will be if it is constructed during developing outbreaks so the expected damage/defoliation can be accurately forecasted to help in reaching control decisions.

Table 3. Douglas-fir tussock moth larval emergence, percent parasitism, and level of naturally-occurring virus infection from egg masses collected in Oregon and Idaho in the fall of 1999 and determined in Victoria, BC, April 2000

OREGON

Coding	Location	No. egg masses reared	Total no. eggs reared[a]	Total no. larvae emerged	Percent larval emergence	No. eggs parasitized by Trich.[b]	Percent parasitism by Trich.[b]	No. eggs parasitized by Tele.[c]	Percent parasitism by Tele.[c]	**Total % Parasitism**	Total No. larvae reared	No. Missing[d]	No. Dead Larvae[e]	% Larval Mortality	No. Dead Larvae with NPV	% Dead Larvae with NPV[f]	**Est. % NPV Infection[g]**
OR1	Fish Creek	34	1443	1147	**79.5**	2	**0.2**	0	0.0	**0.1**	854	6	46	5.4	12[h]	26.1	**1.4**
OR2	Pine Rd. East	17	722	639	**88.5**	0	**0.0**	0	0.0	**0.0**	429	4	32	7.5	1	3.1	**0.2**
OR3	Pine Rd.	3	120	106	**88.3**	0	**0.0**	0	0.0	**0.0**	75	1	1	1.4	0	0.0	**0.0**
OR4	Wallow-Whitman	6	320	227	**70.9**	0	**0.0**	0	0.0	**0.0**	150	1	11	7.4	1	9.1	**0.7**
Total		60	2605	2119	**81.3**	2	**0.1**	0	0.0	**0.1**	1508	12	90	6.0	14	15.6	**0.9**

IDAHO

Coding	Location	No. egg masses reared	Total no. eggs reared[a]	Total no. larvae emerged	Percent larval emergence	No. eggs parasitized by Trich.[b]	Percent parasitism by Trich.[b]	No. eggs parasitized by Tele.[c]	Percent parasitism by Tele.[c]	**Total % Parasitism**	Total No. larvae reared	No. Missing[d]	No. Dead Larvae[e]	% Larval Mortality	No. Dead Larvae with NPV	% Dead Larvae with NPV[f]	**Est. % NPV Infection[g]**
ID9	9	14	600	511	**85.2**	2	**0.4**	0	0.0	**0.3**	351	5	20	5.8	0	0.0	**0.0**
ID12	12	15	603	550	**91.2**	0	**0.0**	0	0.0	**0.0**	377	5	15	4.0	0	0.0	**0.0**
ID117	117	9	404	350	**86.6**	0	**0.0**	0	0.0	**0.0**	225	6	7	3.2	0	0.0	**0.0**
ID216	216-117-2	10	401	376	**93.8**	0	**0.0**	0	0.0	**0.0**	250	1	12	4.8	0	0.0	**0.0**
Total		48	2008	1787	**89.0**	2	**0.1**	0	0.0	**0.1**	1203	17	54	4.6	0	0.0	**0.0**

[a] About 40 eggs per egg mass were reared. The remaining eggs were kept for the bioassaying for the effects of carrier 038® on foliage.

[b] *Trichogramma minutum* complex

[c] *Telenomus californicum*

[d] Sum of number of larvae killed by handling and number of larvae unaccounted for after assay was completed.

[e] Smears were made of **all** dead larvae for virus determination.

[f] Value calculated by formula (No. smears with NPV/No. dead larvae examined) x 100

[g] Value calculated by formula (No. smears with NPV/No. larvae examined) x 100

[h] Eleven of the 12 larvae from OR1 identified as having NPV came from the same egg mass.